CCQM-K159
Free Amino Acids in Plasma

Key Comparison
Track A

Final Report
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SUMMARY

Inherited metabolic disorders affect approximately 1 in 1000 babies; amino acid disorders are less frequent, at approximately 1 in 6000. Babies diagnosed with amino acid disorders require constant monitoring of amino acid blood concentration, especially for disease states such as phenylketonuria. In addition, amino acid measurement may aid in the evaluation of several other disorders such as neurological and nutritional disorders. Free amino acids in healthy patients are typically in the low mg/kg range but, for example, elevated phenylalanine indicates phenylketonuria disease state. Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

This Track A study is being used to assess the core competencies of the National Metrology Institutes/Designated Institutes (NMIs/DIs) for provision of measurement services. The aim of this study is to assess the performance of the NMIs/DIs for a matrix material for clinical analytes. This follows and builds on the successful K109 study on Urea and Uric Acid in Human Serum.

Eleven NMIs participated in the Track A Key Comparison CCQM-K159 Free Amino Acids in Plasma. NMIs/DIs were given the option of taking part in a parallel pilot study P202, however all participants opted for the Track A Key Comparison and there were no participants for CCQM-P202. Participants were requested to evaluate the mass fractions, expressed in mg/kg, of DL-phenylalanine and DL-leucine in pooled frozen human plasma with lithium heparin added as an anticoagulant.

Successful participation in CCQM-K159 demonstrates the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

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ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	calibration and measurement capability
CRM	certified reference material
CV	coefficient of variation, expressed in %: $CV = 100 \cdot s/\bar{x}$
DI	designated institute
DoE	degrees of equivalence
GCHRMS	gas chromatography with high-resolution mass spectrometry detection
GC-IT-MS	gas chromatography with ion trap mass spectrometry detection
GC-MS	gas chromatography with mass spectrometry detection
GC-MS/MS	gas chromatography with tandem mass spectrometry detection
GC-TOFMS	gas chromatography with time-of-flight mass spectrometry detection
LCHRMS	liquid chromatography with high-resolution mass spectrometry detection
LC-MS/MS	liquid chromatography with tandem mass spectrometry detection
ID	isotope dilution
IDMS	isotope dilution mass spectrometry
JCTLM	Joint Committee for Traceability in Laboratory Medicine
KC	key comparison
KCRV	key comparison reference value
LC	liquid chromatography
MADe	median absolute deviation from the median (MAD)-based estimate of s: $MADe = 1.4826 \cdot MAD$, where $MAD = median(\lvert x_i - median(x_i) \rvert)$
MRM	multiple reaction monitoring
NMI	national metrology institute
NMR	nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
pKow	logarithm of the octanol-water partition coefficient
PSE	pressurized solvent extraction
qNMR	quantitative nuclear magnetic resonance spectroscopy
QuEChERS	“quick, easy, cheap, effective, rugged, safe” liquid/solid extraction
RMP	reference measurement procedure
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	selected reaction monitoring

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i/\text{KCRV}$
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
s	standard deviation of a series of quantity values: $s = \sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2/(n-1)}$
t_s	student's *t*-distribution expansion factor
$u(x_i)$	standard uncertainty of quantity value x_i
$\bar{u}(x)$	pooled uncertainty: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i)/n}$
$U(x)$	expanded uncertainty
$U_{95}(x)$	expanded uncertainty defined such that $x \pm U_{95}(x)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
$U_{k=2}(x)$	expanded uncertainty defined as $U_{k=2}(x) = 2 \cdot u(x)$
x	a quantity value
x_i	the i^{th} member of a series of quantity values
$\bar{x}$	mean of a series of quantity values: $\bar{x} = \sum_{i=1}^{n} x_i/n$

INTRODUCTION

Inherited metabolic disorders affect approximately 1 in 1000 babies; amino acid disorders are less frequent at approximately 1 in 6000. Babies diagnosed with amino acid disorders require regular monitoring of free amino acid concentrations in blood, especially for a disease such as phenylketonuria. In addition, amino acid measurement may aid in the evaluation of several other disorders such as neurological and nutritional disorders. Free amino acids in healthy patients are typically in the low mg/kg range but, for example, elevated phenylalanine indicates phenylketonuria disease state. Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

Extraction, clean-up, separation, and quantification of targets in complex biological matrices are competencies demonstrated in this study. Determination of mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine are core challenges for reference material producers and providers of calibration services.

In October 2019, the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) approved the Key Comparison (KC) CCQM-K159 Free Amino Acids in Plasma. NMIs/DIs were given the option of taking part in a parallel pilot study P202, however all participants opted for the Track A Key Comparison and there were no participants for CCQM-P202. CCQM-K159 was designed to assess participants' capabilities for value assignment of a matrix material for clinical analytes. This follows and builds on the successful K109 study on Urea and Uric Acid in Human Serum. [1]

The following sections of this report document the timeline of CCQM-K159, the measurands, study material, participants, results, and the measurement capability claims that participation in CCQM-K159 can support. The Appendices reproduce the official communication materials and summaries of information about the results provided by the participants.

[1] Liu, Q., Liu, H., Chen, Y., Yong, S., Teo, H.L., Wong, L., Teo, T.L., Vamathevan, V., Rego, E.C.P. do, Wollinger, W., Fernandes, J.L.N., Monteiro, T.M., Garrido, B.C., Violante, F.M., Shi, L.H., He, H.H., Quan, C., Xu, B., Li, H.M. and Dai, X.H. (2019). High polarity analytes in biological matrix: determination of urea and uric acid in human serum (CCQM-K109). *Metrologia*, [online] 56(1A), p.08006. doi:https://doi.org/10.1088/0026-1394/56/1a/08006.

TIMELINE

Table 1. Timeline for CCQM-K159

Date	Action
March 2019	Proposed to CCQM
April 2019	Draft protocol presented to OAWG as potential Track A Key Comparison
October 2019	OAWG authorized CCQM-K159 as a Track A Key Comparison; protocol approved
February 2020	Call for participation to OAWG members
August 2020	Study samples were shipped to participants
March 2021	Results due to coordinating laboratory
November 2023	Draft A report distributed to OAWG
October 2024	Draft B report distributed to OAWG
TBD	Final report approved by OAWG

MEASURANDS

The measurands for this study were the free amino acids, DL-leucine and DL-phenylalanine within the mass fraction range of 1-1000 mg/kg. The matrix was a frozen pooled human plasma (anticoagulant - lithium heparin). Figure 1 below displays the molecular structure of these compounds.

DL-leucine
Leu
pK_{OW} -1.743

DL-phenylalanine
Phe
pK_{OW} -1.496

Figure 1. Structures of analytes

STUDY MATERIALS

The study materials were sourced from LGC Maine Standards (Maine, USA). A pooled frozen human plasma with lithium heparin added as an anticoagulant, was supplied as 1000 units of 1.2 mL, prepared from a homogenized material. The units were supplied in screw-capped cryo safe vials, in vial boxes that were stored at -80 °C on arrival and until distribution for the study.

Homogeneity Assessment of Study Material

Homogeneity was assessed by analysis of 250 mg aliquots of 15 units in triplicate, analysed in three separate batches. Statistical analysis of this data is summarized in Table 2, and plots of the individual results are shown for DL-leucine in Figure 2, and DL-phenylalanine in Figure 3.

Within unit and between unit variance was assessed with a one-way ANOVA and found to be acceptable. Typical measurement uncertainty for individual measurements was <1 %.

Table 2. Results of the homogeneity assessment for leucine and phenylalanine in plasma.

ANOVA Estimate	Leucine	Phenylalanine
Within-unit, CV_{wth}:	0.39 %	0.39 %
Between-unit, CV_{btw}:	0.56 %	0.52 %
Total analytical variability, CV:	0.76 %	0.84 %

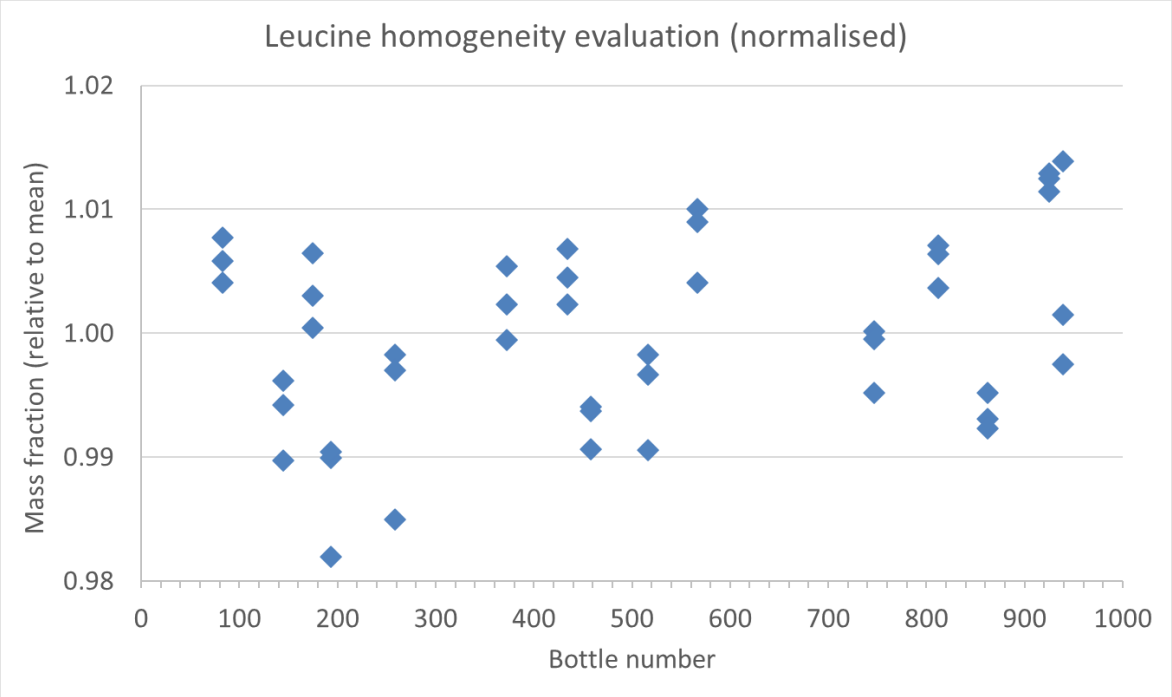


Figure 2. Homogeneity evaulation for leucine

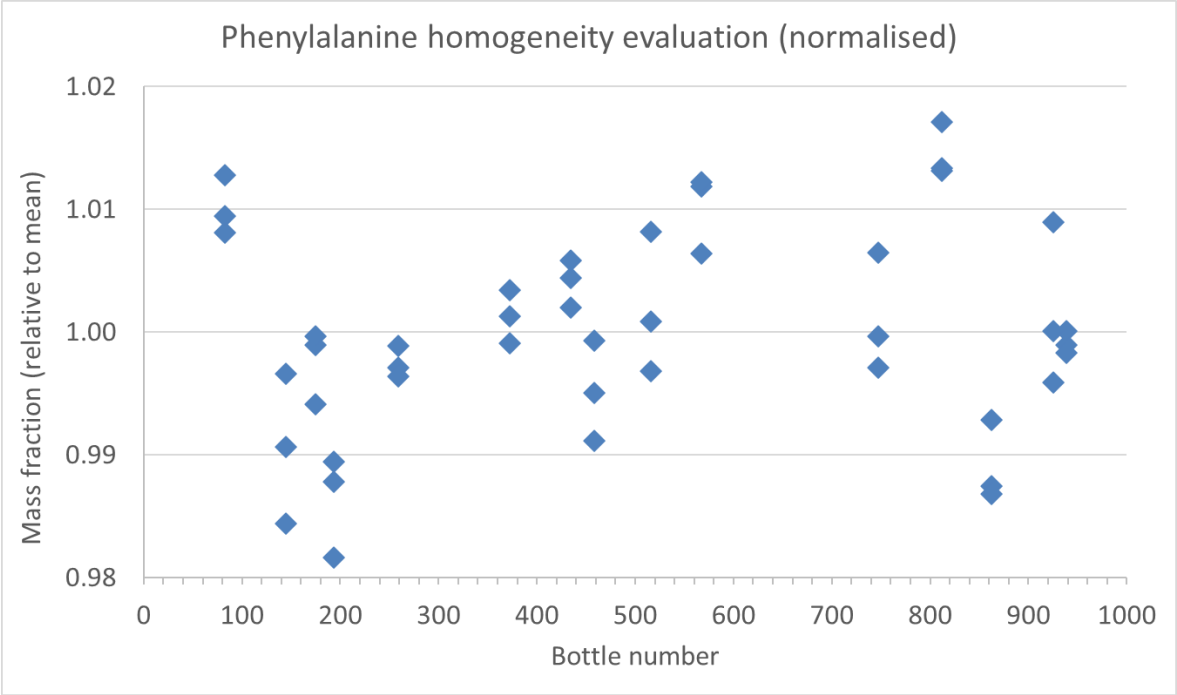


Figure 3. Homogeneity evaluation for phenylalanine

Stability Assessment of Study Material

Short term stability was assessed by analysis of two units in duplicate which had been stored under four conditions, namely nominal temperatures of -80 °C, –20 °C, 4 °C and 18 °C. Time points of T0, 1 week, 2 weeks and 4 weeks were analysed as the materials became available i.e. non-isochronously. Results for DL-leucine and DL-phenylalanine are shown in Figures 4 and 5 respectively. Some instability was observed at 4 °C and 18 °C, however at -20 °C and -80 °C no significant change was observed.

Long term stability was assessed also by non-isochronous analysis after 3-, 6- and 9-months storage at -20 °C and -80 °C. Results of -80 °C storage are shown in Figures 6 and 7. No significant change in concentration was found over the time period.

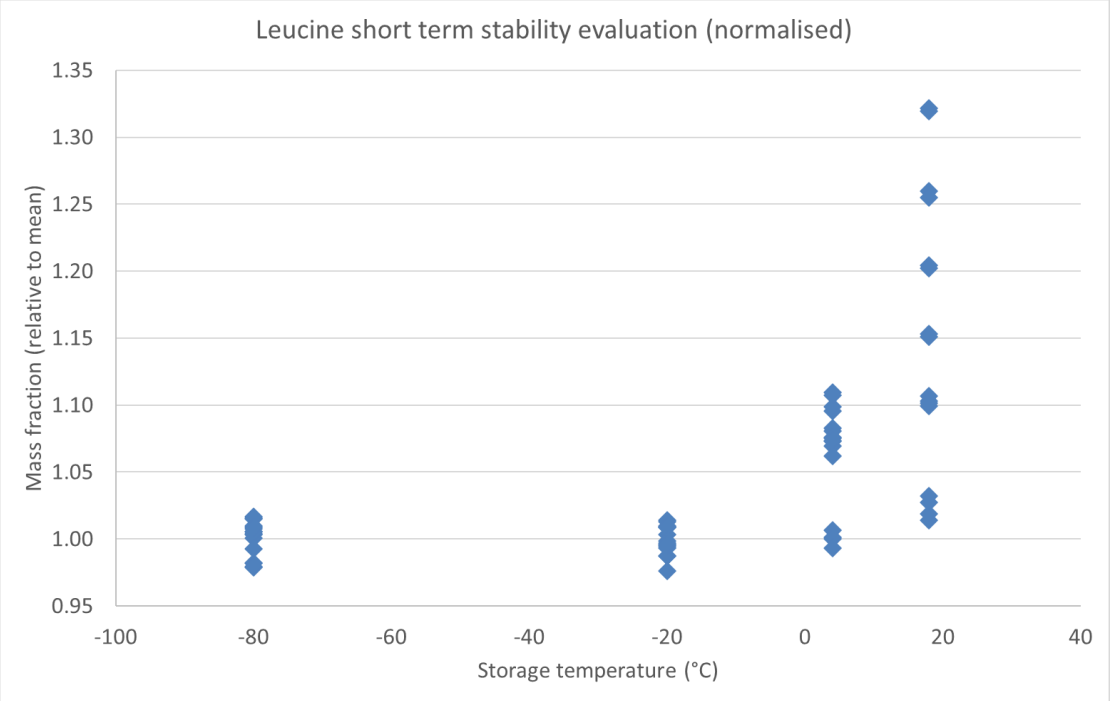


Figure 4. Short term stability evaluation for leucine

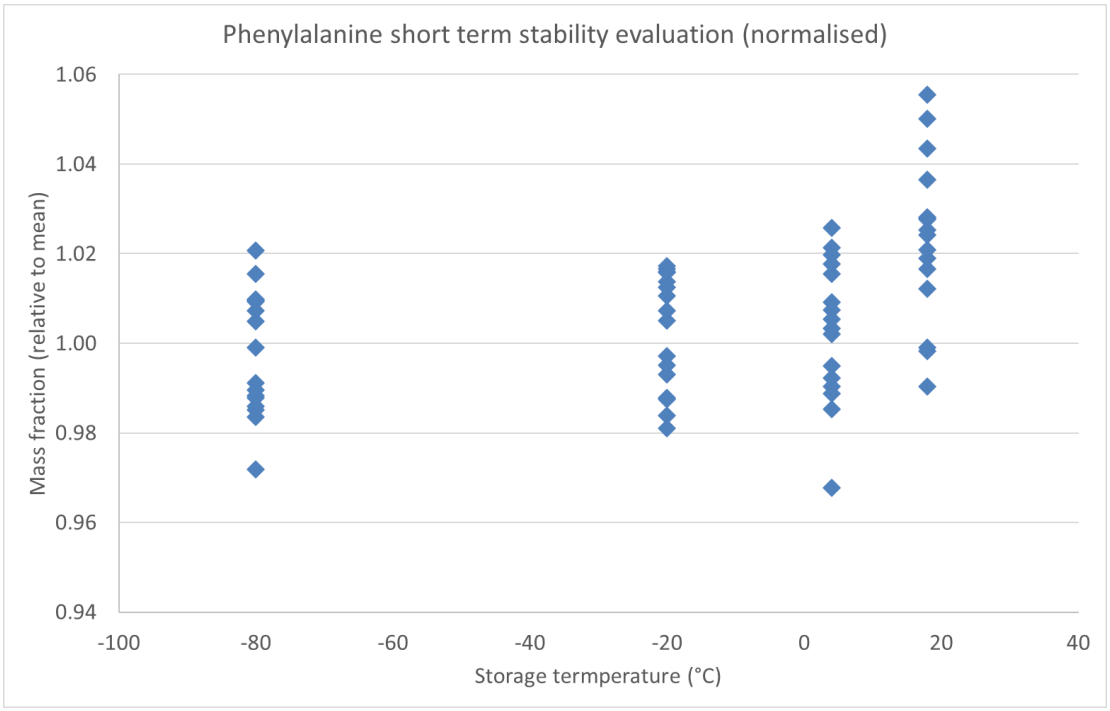


Figure 5. Short term stability evaluation for phenylalanine

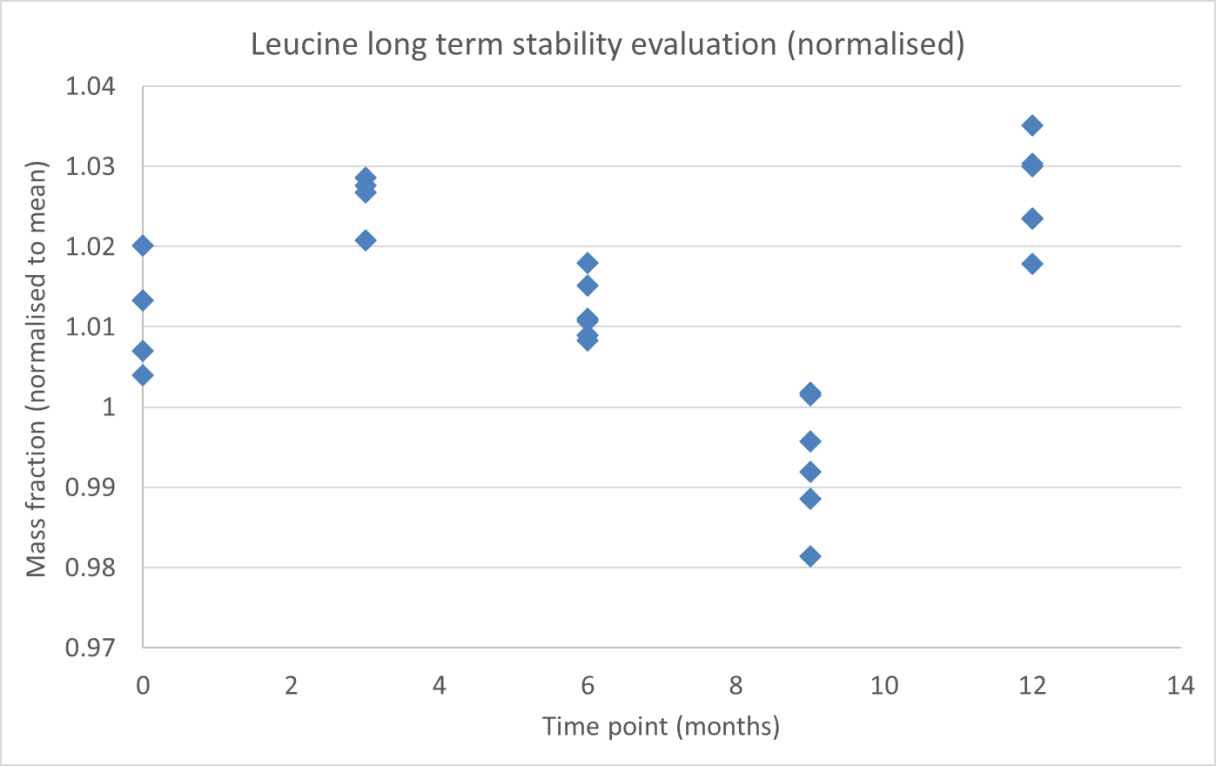


Figure 6. Long term stability evaluation for leucine

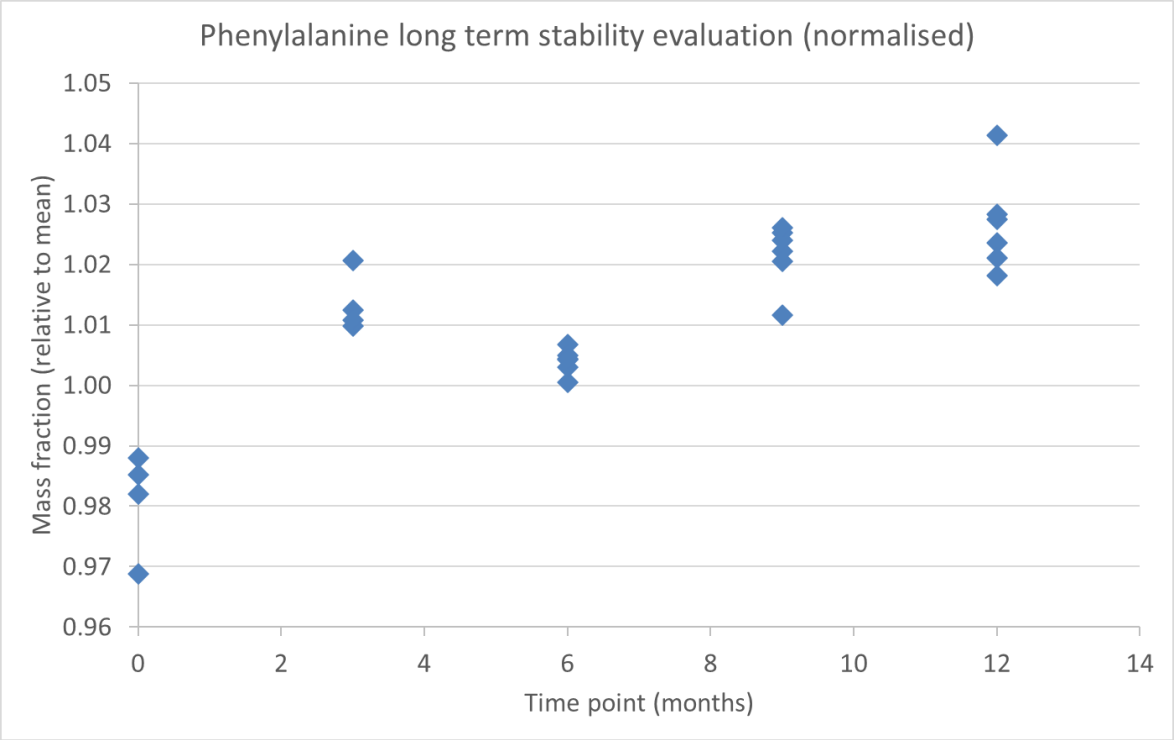


Figure 7. Long term stability evaluation for phenylalanine

The long term stability (LTS) data were subject to the same statistical analysis as the homogeneity data set for comparison, Table 3 shows the results from this analysis in comparison to the original homogeneity data set.

Table 3. Results of the LTS data analysed as homogeneity data in comparison to the original homogeneity assessment data for free DL-leucine and DL-phenylalanine in frozen human plasma

Analyte	**Study**	**Between-unit SD**	**Within-unit SD**
Leucine	Homogeneity	0.56 %	0.39 %
	LTS as homogeneity	0.69 %	0.34 %
Phenylalanine	Homogeneity	0.53 %	0.39 %
	LTS as homogeneity	0.76 %	0.44 %

PARTICIPANTS, INSTRUCTIONS AND SAMPLE DISTRIBUTION

The call for participation in the study was issued in February 2020 with the intent to distribute samples in August 2020, receive results in February 2021, and discuss results at the online OAWG meeting in April 2021. See Table 4 for a list of the institutes that registered for CCQM-K159. Appendix A reproduces the Call for Participation; Appendix B reproduces the study Protocol.

Table 4. Institutes registered for CCQM-K159

NMI or DI	Code	Country	Contact
National Measurement Institute Australia	NMIA	Australia	Mark Lewin
National Metrology Institute of Japan	NMIJ / AIST	Japan	Tomoya Kinumi
Health Sciences Authority	HSA	Singapore	Qinde Liu
Instituto Nacional de Metrologia, Qualidade e Tecnologia	INMETRO	Brazil	Eliane C. P. Rego / Wagner Wollinger
National Metrology Institute of Türkiye/TÜBITAK Ulusal Metroloji Enstitüsü	UME	Türkiye	Mine Bilsel
D.I. Mendeleyev Research Institute for Metrology	VNIIM	Russian Federation	Anatoliy Krylov / Alena Mikheeva
National Institute of Metrology Thailand	NMIT	Thailand	Jintana Nammoonnoy
Korea Research Institute of Standards and Science	KRISS	Korea (Republic of)	Ha-Jeong Kwon, Ji-Seon Jeong
Physikalisch-Technische Bundesanstalt	PTB	Germany	Andre Henrion
National Laboratory of Chemical Metrology/General Chemistry State Laboratories - Hellenic Institute of Metrology	EXHM/GCSL-EIM	Greece	Elias Kakoulides
LGC Ltd.	LGC	United Kingdom	Emily Whyte
National Institute of Metrology	NIM	China	Can Quan
National Metrology Institute of South Africa	NMISA	South Africa	Desiree Prevoo-Franzsen

Call for participation was completed in February 2020. The intention was to ship samples within five weeks of the call for participation ending, however sample distribution was severely delayed due to the SARS-CoV-2 global pandemic. Samples were eventually shipped during August and September 2020. Samples were shipped on dry ice with temperature monitors inside the packages to confirm all packages remained frozen (< 0 °C) upon arrival at their destinations.

Each participant received five units of material, selected at random and packed into individual boxes. The recommended minimum sample amount for analysis was 250 mg and measurement results were to be reported on an as-received basis.

Participants requested a longer time period to report results due to procurement of standards and staffing issues caused by the SARS-CoV-2 global pandemic; therefore, the results submission deadline was extended to the 31st March, 2021. The preliminary results were presented at the online OAWG video conference meeting on the 5th May 2021.

The National Metrology Institute of South Africa (NMISA) withdrew from the study in May 2020 due to the requirement to prioritise many other projects that were delayed due to the SARS-CoV-2 pandemic. The National Institute of Metrology (NIM) withdrew in January 2021 after failure to obtain customs approval for receipt of the samples due to the SARS-CoV-2 pandemic. The samples were not sent to these participants.

Participants were requested to report a single estimate of the mass fraction in units of mg/kg for DL-phenylalanine, and to report individual unit measurements for both DL-leucine and DL-phenylalanine. The reported mass fraction for DL-phenylalanine was to be the overall mean from measurements from three separate units with the standard uncertainty, expanded uncertainty at 95 % level of confidence and the coverage factor.

In addition to the quantitative results, participants were requested to describe their analytical methods, including details of the calibrants and their traceability, internal standards, any QC materials, calculation of results, and uncertainty budgets, as well as the Core Competencies they felt were demonstrated in this study.

RESULTS

Alongside the request for estimates of the mass fraction (mg/kg) for both DL-leucine and DL-phenylalanine, participants were asked to provide their analytical methods, approach to uncertainty estimation, and the Core Competencies that were demonstrated. Appendices C, D, and E reproduce the relevant report forms. CCQM-K159 results were received from all of the 11 institutions that received samples.

Participants were initially requested to report a single estimate of the mass fraction (mg/kg) for both DL-leucine and DL-phenylalanine. However, it came to light through LGC's own participation in K159 of a potential issue with a subset of units. The samples may have degraded between the initial evaluation and shipment to the participants, affecting the values of DL-leucine. Participants were asked to continue to analyse the materials but asked that they only report results for each of the three individual units for DL-leucine, and for each of the three units and a combined value for DL-phenylalanine. Participants were informed of the change to reporting by email in March 2021 prior to the reporting deadline. Consequently, results discussed in this report are only associated with DL-phenylalanine measurements, including the KCRV estimations.

Calibration Materials Used by Participants

Participants established the metrological traceability of their results using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they had determined the purity in house. Table 5 lists the CRMs that were available for use. Table 6 lists how participants established traceability. If through their own measurements, Table 6 also lists the material, its assigned purity, the method used for purity evaluation, and how the participant had demonstrated their competence in the use of the method(s).

Table 5. Certified Reference Materials available for use

CRM	Provider	Analyte	Mass Fraction[a] Delivered, mg/g	Mass Fraction[a] Source Material, mg/g	In-house Purity Methods Used to Value-Assign Source Material
CRM 6012-a	NMIJ	Leu	N/A	999 ± 2	Titration, LC-MS, TGA, Karl Fischer
CRM 6014-a	NMIJ	Phe	N/A	999 ± 2	Titration, LC-MS, TGA, Karl Fischer
CRM 2389a	NIST	Leu Phe	0.319 ± 0.014 0.421 ± 0.014	N/A	Gravimetric and LC-MS/MS
HRM 1008A	HSA	Leu	N/A	996.9 ± 3.3	LC-MS, TGA, Karl Fischer
HRM 1014A	HSA	Phe	N/A	997.5 ± 3.6	LC-MS, TGA, Karl Fischer

a Stated as Value ± U_{95}(Value)

Table 6. Metrological traceability of participants' results

NMI/DI	Analyte	Source of Traceability	Material	Mass Fraction[a] Purity, %	Purity Techniques[b]	Evidence of Competence
NMIA	Phe Leu	CRM 6014-a CRM 6012-a	N/A			
NMIJ	Phe Leu	CRM 6014-a CRM 6012-a	N/A			
HSA	Phe Leu	HRM-1014A HRM-1008A	N/A			
INMETRO	Phe Leu	INMETRO	Sigma Aldrich Sigma Aldrich	99.902 ± 0.095 99.86 ± 0.1	qNMR	INMETRO has broad CMC for purity assessment "Mass fraction purity of organic compounds of high polarity (pKOW > -2) with molar mass below 500 g/mol". K55d DoE (0,7%) used combination of mass balance and qNMR.
UME	Phe Leu	UME	Acros Organics Acros Organics	99.70 ± 0.23 99.63 ± 0.23	qNMR	The capability is underpinned by participating in CCQM-K55b-d, CCQM-K104 and CCQM-K148.a comparisons.
VNIIM	Phe Leu	VNIIM	Sigma Aldrich Sigma Aldrich	99.62 ± 0.03 99.75 ± 0.03	Mass balance LC/MS, LC/LS, GC/MS, LC-DAD, GC/FID, ICP/MS, TGA, KF titration	CMC (High purity chemicals - phenylalanine, leucine) approved 2019-10-23.
NMIT	Phe Leu	CRM 6014-a CRM 6012-a	N/A			

NMI/DI	Analyte	Source of Traceability	Material	Mass Fraction[a] Purity, %	Purity Techniques[b]	Evidence of Competence
KRISS	Phe Leu	CRM 6014-a CRM 6012-a	N/A			
PTB	Phe Leu	CRM 6014-a CRM 6012-a	N/A			
EXHM/GCSL-EIM	Phe Leu	EXHM/GCSL-EIM	Commercially available amino acids in solid form (purity determined in-house with qNMR against NMIJ CRM 4601a)	99.91 ± 0.22 99.77 ± 0.21	qNMR The gravimetrically assigned values of the calibration solutions were also assessed by LC-IDMS against NIST SRM 2389a	EXHM has participated in the following CCQM purity comparisons: P150, P150b, K148a, K148b, P117c and CCQM-K104. EXHM has also participated in CCQM-K154a, b and d on the preparation and value assignment of mycotoxin solutions. According to their HFTLS statements, these comparisons provide evidence for purity value assignment when the participants have used with their our own calibrants, and not the solutions provided by BIPM, which is the case for EXHM.
LGC	Phe Leu	CRM 6014-a CRM 6012-a	N/A			

a Stated as Value ± U_{95}(Value)

b DSC: Differential scanning calorimetry
GC-FID: Gas chromatography with flame ionization detection
HPLC-DAD: High performance liquid chromatography with diode-array detection
MB: Mass balance
qNMR: Quantitative nuclear magnetic resonance

Methods Used by Participants

Table 7. Summary of the methods used by participants in CCQM-K159

NMI/DI	Approximate sample size used (g)	Phenylalanine Internal Standard Labelling	Extraction	Instrument Technique	Calibration Strategy	Calibration method
NMIA	0.200	13C9, 15N	Protein precipitation, dansyl chloride derivatisation	2D LC-MS/MS	DEM-IDMS	single point calibration, bracketed
NMIJ/	0.250	13C9, 15N	Protein precipitation, N-butylnicotinic acid N-hydroxysuccinimide ester derivatisation	LC-MS/MS	IDMS	5-point calibration curve
HSA	0.250	13C6	Protein precipitation, N-tert-butyldimethylsilyl-N-methyl-trifluoroacetamide derivatisation for GC method	LC-MS/MS and GC-MS	IDMS	4-point calibration curve
INMETRO	0.300	D5	Protein precipitation	LC-MS/MS	IDMS	5-point internal calibration curve

UME	0.250	D5	Liquid/liquid extraction, propyl chloroformate derivatisation	LC-Orbitrap	IDMS	5-point calibration curve
VNIIM	0.250	13C9, 15N	Protein precipitation	LC-MS/MS	IDMS	single point calibration by 3 calibration solutions
NMIT	0.250	13C9, 15N	Protein precipitation	LC-MS/MS	IDMS	single point calibration
KRISS	0.500	13C9, 15N	Protein precipitation	LC-qTOF	IDMS	exact matched single point calibration
PTB	0.250	13C9, 15N	Protein precipitation, Liquid/liquid extraction	LC-MS/MS	IDMS	single point calibration
EXHM/GCSL-EIM	0.250	15N	Protein precipitation	LC-MS/MS	IDMS	exact matched single point calibration
LGC	0.250	13C9, 15N	Protein precipitation, MTBSTFA derivatisation	GC-MS/MS	DEM-IDMS	exact matched single point calibration, bracketed

Full descriptions of the analytical methods used by the participants, including sample preparation, analytical technique, and quantification approach are summarized in Appendix F. The participants' approaches to estimating uncertainty are provided in Appendix G.

Participant Results for DL-phenylalanine

The results for CCQM-K159 for the determination of DL-phenylalanine are detailed in Table 8 and presented graphically in Figure 8.

Table 8. Reported results for DL-phenylalanine

NMI	DL-Phenylalanine mg/kg					
	x	$u(x)$	$u(x)$ %	k	$U(x)$	$U(x)$ %
NMIA	61.40	0.88	1.4	2.13	1.90	3.1
NMIJ / AIST	60.10	0.32	0.5	2.00	0.64	1.1
HSA	60.81	0.83	1.4	2.00	1.66	2.7
INMETRO	60.70	1.30	2.1	2.00	2.60	4.3
UME	57.65	2.06	3.6	2.00	4.12	7.1
VNIIM	58.50	1.70	2.9	2.00	3.40	5.8
NMIT	60.30	1.20	2.0	2.00	2.40	4.0
KRISS	61.48	0.70	1.1	2.20	1.53	2.5
PTB	60.45	0.67	1.1	2.00	1.30	2.2
EXHM/GCSL-EIM	59.74	2.16	3.6	2.00	4.32	7.2
LGC	59.59	0.72	1.2	2.00	1.45	2.4
n	11					
$\bar{x}$	60.07					
s	1.16					
CV %	1.93					

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation;
CV = $100 \cdot s/\bar{x}$

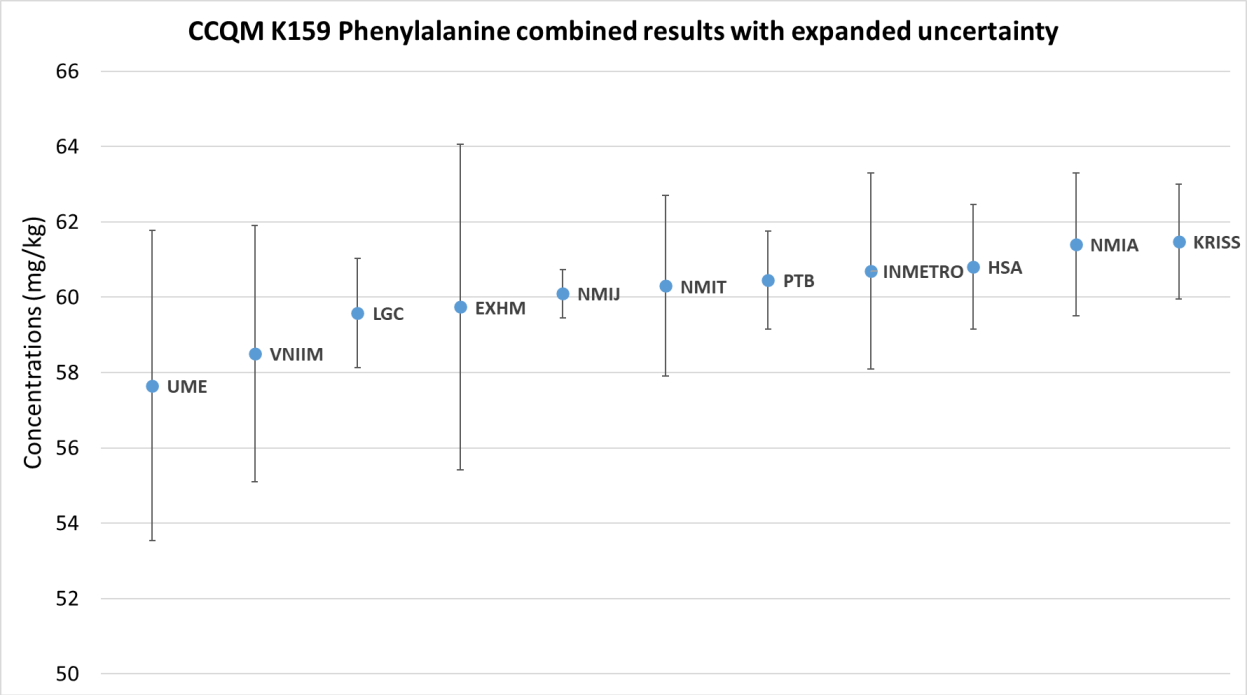


Figure 8. Illustrated reported results for phenylalanine.

Results are sorted by increasing reported value. Data points represent the reported values, error bars represent ± their expanded uncertainties, U(x). The thin horizontal gridlines are provided for visual guidance.

Overall, the results show good agreement between the institutes for the measurement of DL-phenylalanine in plasma, with the reported expanded uncertainties of all institutes overlapping with each other.

DL-PHENYLALANINE KEY COMPARISON REFERENCE VALUE (KCRV)

The KCRV was computed using the institute means and standard uncertainties using a number of different methods. Degrees of equivalence, d_i , were determined as:

$$d_i = \bar{x}_i - \hat{\mu}$$

Equation 1.

where $\hat{\mu}$ is the selected KCRV. The uncertainty in d_i is:

$$u^2(d_i) = u^2(\bar{x}_i) + u^2(\hat{\mu}) - 2cov(\bar{x}_i, \hat{\mu})$$

Equation 2.

Selected candidate KCRVs and their standard uncertainties are listed in Table 9. Only those estimators which do not require additional variance components are included. Both weighted and unweighted estimates have been calculated, as there is no reason to conclude that the institute uncertainties vary in their reliability. The results of all estimators are very similar to each other.

Table 9. Candidate KCRVs for DL-phenylalanine, including both weighted and unweighted methods. Robust estimates are included for comparison purposes but are not considered optimum as there are no outliers in the dataset. For the Huber estimate, a tuning constant of 1.345 was used to obtain 95% efficiency.

	KCRV (mg/kg)	u **(mg/kg)**	**n**	**k**	U **(mg/kg)**
Arithmetic mean	60.065	0.351	11	2.228	0.782
Weighted mean	60.322	0.219	11	2.228	0.489
Median	60.300	0.314	11	2.228	0.699
Huber Proposal 2	60.165	0.363	11	2.228	0.810

These candidate KCRVs are shown graphically in Figures 9, 10, 11 and 12.

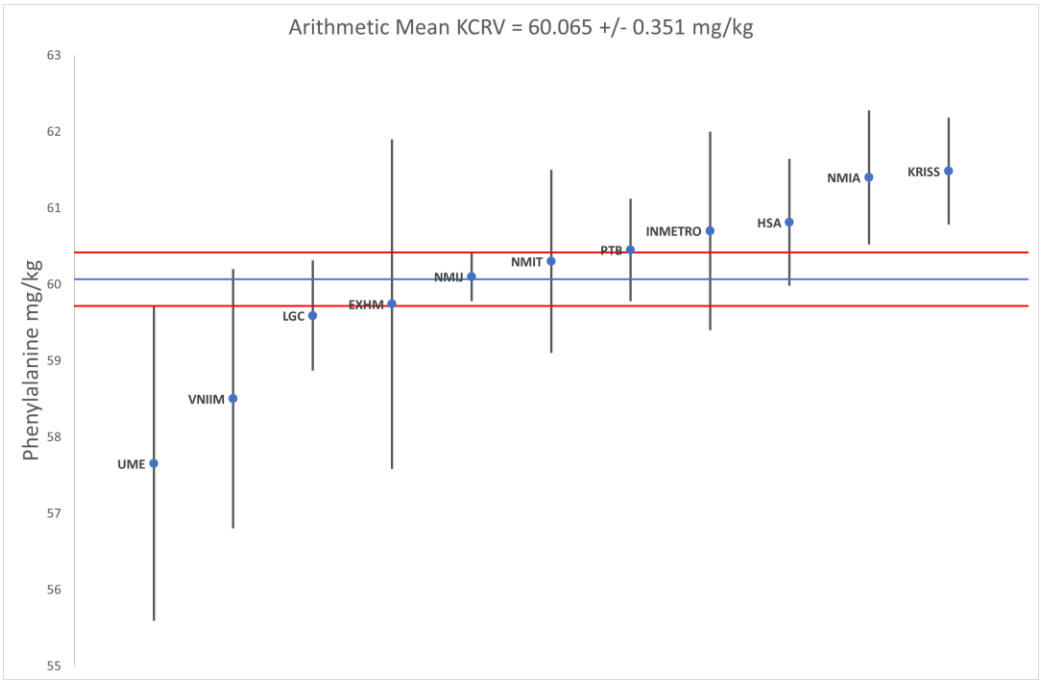


Figure 9. The candidate KCRV based on the arithmetic mean.

The results are sorted by increasing reported value. Data points represent the reported mean values, x; error bars denote the standard uncertainties, u(x). The blue horizontal line denotes the candidate KCRV. The bracketing red lines denote the standard uncertainty of the candidate KCRV.

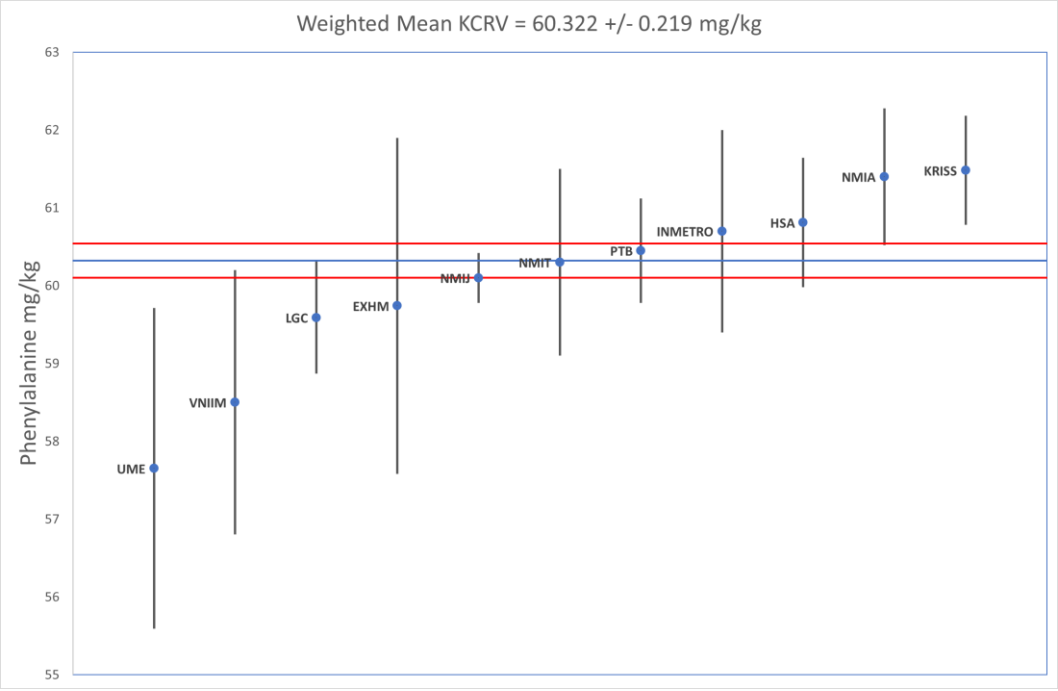


Figure 10. The candidate KCRV based on the weighted mean.

The results are sorted by increasing reported value. Data points represent the reported mean values, x; error bars denote the standard uncertainties, u(x). The blue horizontal line denotes the candidate KCRV. The bracketing red lines denote the standard uncertainty of the candidate KCRV.

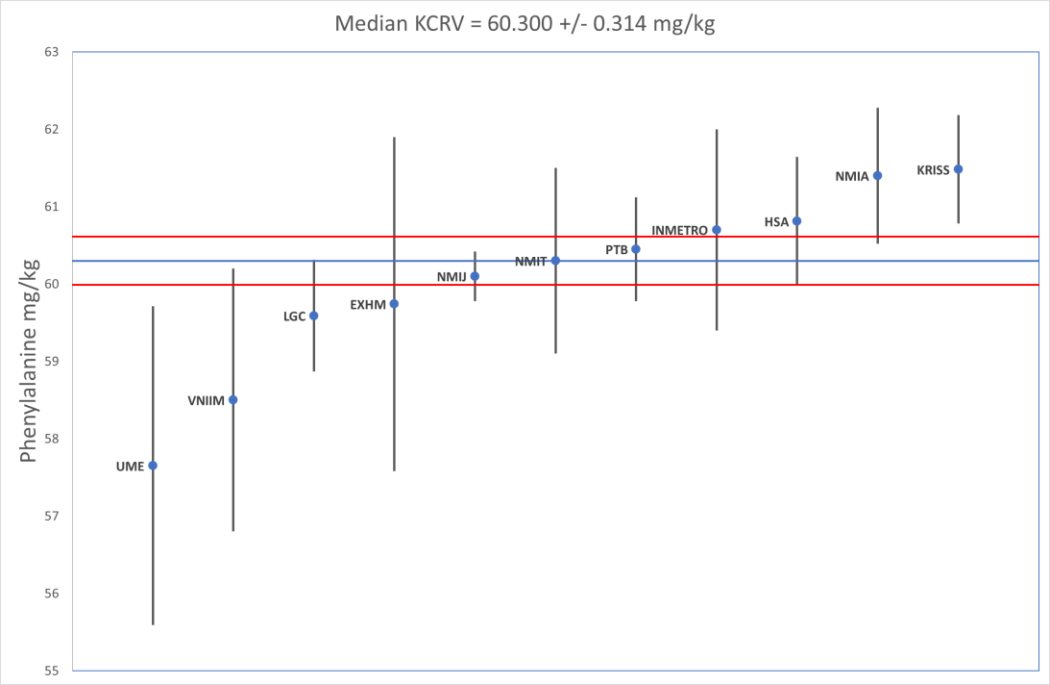


Figure 11. The candidate KCRV based on the median.

The results are sorted by increasing reported value. Data points represent the reported mean values, x; error bars denote the standard uncertainties, u(x). The blue horizontal line denotes the candidate KCRV. The bracketing red lines denote the standard uncertainty of the candidate KCRV.

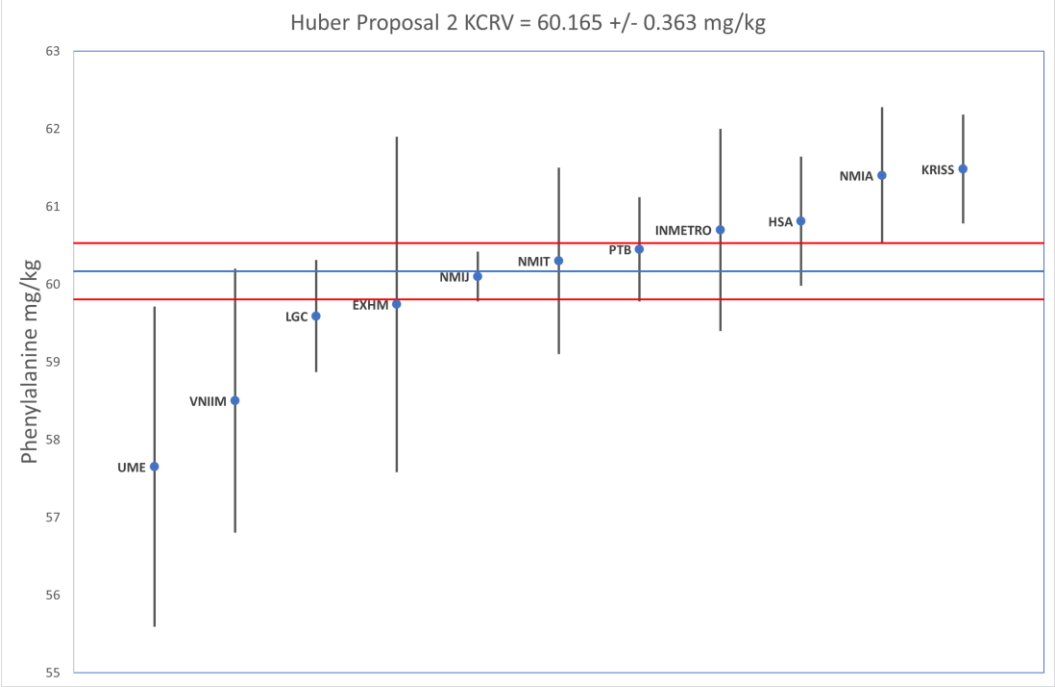


Figure 12. The candidate KCRV based on the Huber Proposal 2.

The results are sorted by increasing reported value. Data points represent the reported mean values, x; error bars denote the standard uncertainties, u(x). The blue horizontal line denotes the candidate KCRV. The bracketing red lines denote the standard uncertainty of the candidate KCRV.

In a Key Comparison study, the usual choice for the KCRV is the estimate in the set of appropriate estimators which has minimum variance. In this case the minimum-variance estimate is the uncertainty-weighted mean, where the weights, *wi*, are the inverse squared standard uncertainties $\frac{1}{u_i^2}$ and the standard uncertainty in $\hat{\mu}$ is:

$$u^2(\hat{\mu}) = \frac{1}{\sum_{i=1}^{n} \frac{1}{u^2(\bar{x}_i)}}$$

Equation 3.

It has been demonstrated that the submitted uncertainties are consistent with their respective estimates, and the weighted mean is recommended for use as the KCRV.

DL- PHENYLALANINE DEGREES OF EQUIVALENCE (DoE)

The absolute degrees of equivalence for the participants in CCQM-K159 are estimated as the signed difference between the combined value and the KCRV:

$$d_i = x_i - \text{KCRV}.$$

Equation 4.

Table 10 shows the results with degrees of equivalence calculated using the recommended KCRV (weighted mean) of 60.32 mg/kg. For a consistent dataset, the absolute value of the ratio of the degree of equivalence and its expanded uncertainty with $k = 2$ is expected to be below 1 in 95% of cases.

Table 10. Estimates and degrees of equivalence with respect to the recommended KCRV.

Institute	**Estimate**	u	**Weight**	u**KCRV**	**Covariance**	**DoE**	u**DoE**	**DoE/** U**DoE**
TUBITAK UME	57.65	2.06	0.236	0.219	0.204	-2.672	1.971	-0.68
VNIIM	58.50	1.70	0.346	0.219	0.139	-1.822	1.631	-0.56
LGC	59.59	0.72	1.929	0.219	0.025	-0.732	0.719	-0.51
EXHM	59.74	2.16	0.214	0.219	0.224	-0.582	2.065	-0.14
NMIJ	60.10	0.32	9.766	0.219	0.005	-0.222	0.375	-0.30
NMIT	60.30	1.20	0.694	0.219	0.069	-0.022	1.162	-0.01
PTB	60.45	0.67	2.228	0.219	0.022	0.128	0.674	0.10
INMETRO	60.70	1.30	0.592	0.219	0.081	0.378	1.255	0.15
HSA	60.81	0.83	1.452	0.219	0.033	0.488	0.819	0.30
NMIA	61.40	0.88	1.291	0.219	0.037	1.078	0.865	0.62
KRISS	61.48	0.70	2.041	0.219	0.024	1.158	0.701	0.83

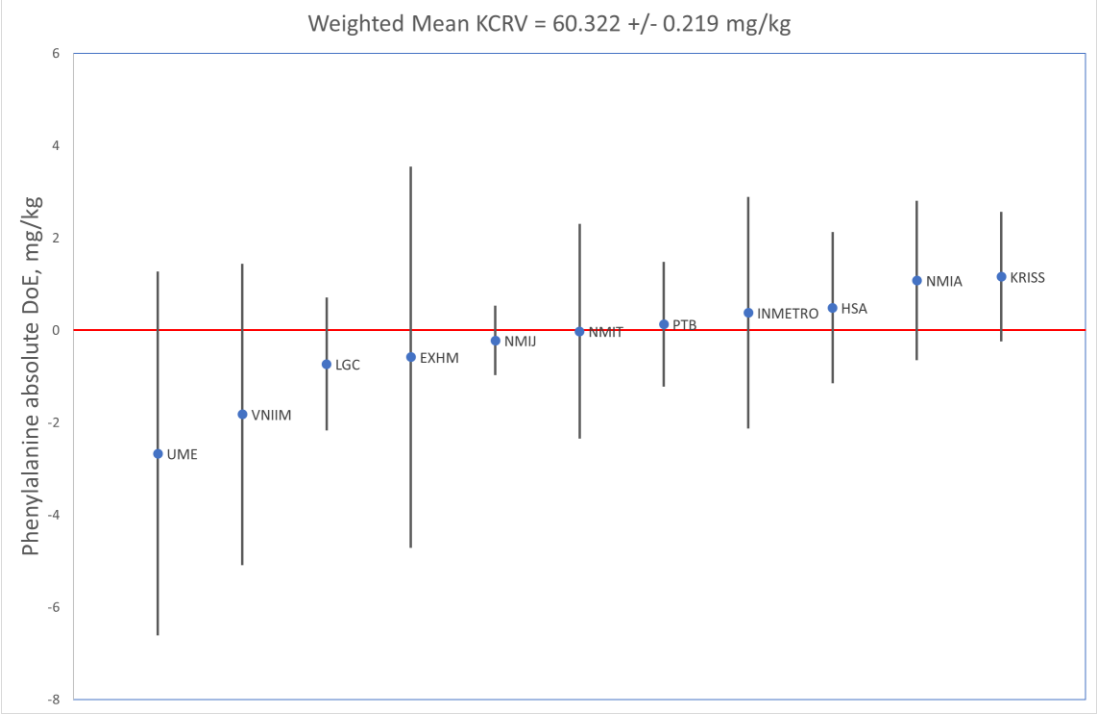


Figure 13. Displays the absolute DoE for the Weighted Mean candidate KCRV for phenylalanine.

The results are sorted by increasing reported value. The axis to the left edge displays the absolute DoE, d, in mg/kg. Data points represent d, and the bars their approximate 95 % expanded uncertainties, U95(d). The red horizontal line denotes a very good agreement with the candidate KCRV.

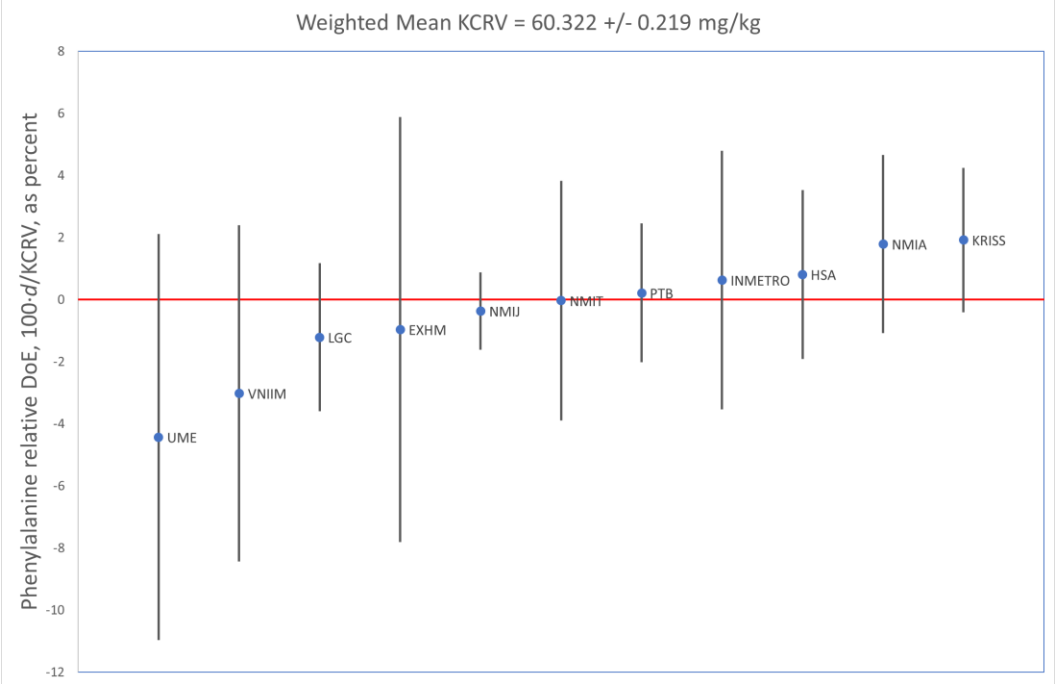


Figure 14. Displays the relative DoE as percent for the Weighted Mean candidate KCRVs for phenylalanine.

The results are sorted by increasing reported value. The axis to the left edge displays the relative DoE, 100·d/KCRV, as percent. Data points represent d, and the bars their approximate 95 % expanded uncertainties, U95(d). The red horizontal line denotes a very good agreement with the candidate KCRV.

USE OF CCQM-K159 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K159 demonstrates the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) clean-up and separation of the analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.

Core Competency Statements and CMC support

Appendix E lists the Core Competencies claimed by the participants in CCQM-K159. The information in these Tables is as provided by the participants. Details of the analytical methods used by each participant in this study are provided in Appendix F.

As all participants' DoE overlap with the candidate KCRV, everyone has demonstrated their competency within their level of uncertainty. Therefore, CMCs that align with the HFTLS, and uncertainties that align with the DoE are supported.

CONCLUSIONS

All participants demonstrated their capability of determining DL-phenylalanine in a biological matrix at mass fraction within 1-1000 mg/kg.

The DL-phenylalanine mass fraction KCRVs (wKCRV) for CCQM-K159 spanned a mass fraction range of 60.065 mg/kg to 60.322 mg/kg. The relative expanded uncertainties U(wKCRV) ranged from 0.81 % to 1.35 %.

Inspection of the degree of equivalence plots (Figures 13 and 14) for the DL-phenylalanine mass fraction assignments in CCQM-K159 indicated that there was an excellent agreement between results for all participants.

ACKNOWLEDGEMENTS

The study coordinators thank the participating laboratories for providing the requested information used in this study.

APPENDIX A: Call for Participation

From: Mackay, Lindsey
Sent: Wednesday, February 05, 2020 9:26 AM
To:
Subject: Call for participation in CCQM-K159 Amino acids in plasma and BIPM Visiting Scientist Opportunities [SEC=UNCLASSIFIED]

Dear OAWG Colleagues

This is the formal call for participation in our next Track A comparison for amino acids in plasma. The key comparison CCQM-K159 is being run in parallel with the pilot study CCQM-P202 so you have both options, but if you would like to submit CMCs related to the scope of this comparison in the future then you will need to participate in the key comparison.

LGC have added in the long term stability data to the protocol and the deadline for submission of results has been shifted to 30 June 2020 due to the delay in getting to this point. Could you please register with LGC using the attached form by 14 February 2020 as they will be aiming to distribute samples by the end of February.

Robert Wielgosz from BIPM has passed on to me the current information on visiting scientist opportunities at BIPM for quarter 4 2020 and for 2021.
The document is attached and is also available from the following link:

https://www.bipm.org/cc/CCQM/Allowed/26/CCQM20-04_BIPM_Chemistry_2020-2021.pdf

Anyone interested, or with colleagues who are interested, are invited to contact Robert for further information (email:).

Best regards
Lindsey

APPENDIX B: Protocol

CCQM-K159/CCQM-P202
Free Amino Acids in Plasma

Key Comparison
Track A

Study Protocol
October 2019

Emily Whyte and Chris Hopley
LGC
Teddington, Middlesex, TW11 0LY, England

INTRODUCTION

This Track A study is being used to assess the core competencies of the National Metrology Institutes/Designated Institutes (NMIs/DIs) for provision of measurement services. The aim of this study is to assess the performance of the NMIs/DIs for a matrix material for clinical analytes. This follows and builds on the successful K109 study on Urea and Uric Acid in Human Serum. Several options were presented to the OAWG, including metanephrines and aldosterone, before it was agreed to progress the study on amino acids in human plasma.

Inherited metabolic disorders affect approximately 1 in 1000 babies, amino acid disorders are less frequent, and approximately 1 in 6000. Babies diagnosed with amino acid disorders require constant monitoring of amino acid levels, especially for disease states such as phenylketonuria. In addition, amino acid measurement may aid in the evaluation of several other disorders such as neurological and nutritional disorders.

Participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine. These are core challenges for reference material producers and providers of calibration services. Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

CCQM-K159 is being co-ordinated in parallel with pilot study CCQM-P202. This protocol also covers the parallel pilot study.

TIMELINE

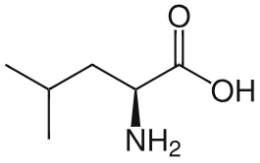
Table 1 lists the timeline for the proposed study.

Table 1: Timeline of CCQM-K159/CCQM-P202

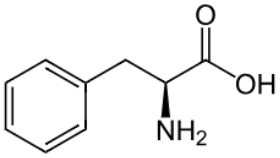
Date	Action
November 2018	Sample Preparation
March – November 2019	Homogeneity and Stability Testing
January 2020	Call for participation to OAWG members
July/August 2020	Sample Distribution
February 2021	Deadline for Submission of Results
April 2021	Preliminary Discussion of Results

MEASURANDS

The measurands for this study are the free amino acids, DL-Leucine and DL-Phenylalanine which are within the concentration range 1-1000 mg/kg. The matrix is frozen pooled human plasma (lithium heparin) which has been screened, the units are 1.2 mL aliquots in screw capped vials and are stored at -80°C. Data is to be reported as received, mass fractions, mg/kg on the combined value of three units.



DL-Leucine
Leu
pK_{OW} -1.743



DL-Phenylalanine
Phe
pK_{OW} -1.496

Figure 1: Structures of target analytes

STUDY MATERIALS

Frozen pooled human plasma (lithium heparin), 1000 units of 1.2 mL aliquots prepared from homogenised material. These are in screw capped cryo safe vials that have been stored at -80C.

Each participant will receive 5 units of 1.2 mL aliquots of frozen pooled human plasma. Measurement results were to be reported on an as-received basis.

Recommended Minimum Sample Amount

The recommended minimum sample amount for analysis is at least 250 µL.

Homogeneity Assessment of Study Material

Homogeneity was assessed by analyzing 250 µL aliquots of 15 units in triplicate across 3 batches. This data is summarized in Table 2, and plots of the individual results are shown for Leucine in Figure 2, and Phenylalanine in Figure 3. The data was assessed with a one-way ANOVA and found to be acceptable.

Table 2. Results of the homogeneity assessment for free DL-leucine and DL-phenylalanine in frozen human plasma

ANOVA Estimate	Leucine	Phenylalanine
Within-packet, CV_{wth}:	0.39 %	0.39 %
Between-packet, CV_{btw}:	0.56 %	0.52 %
Total analytical variability, CV:	0.76 %	0.84 %

Figure 2. Homogeneity evaluation for Leucine

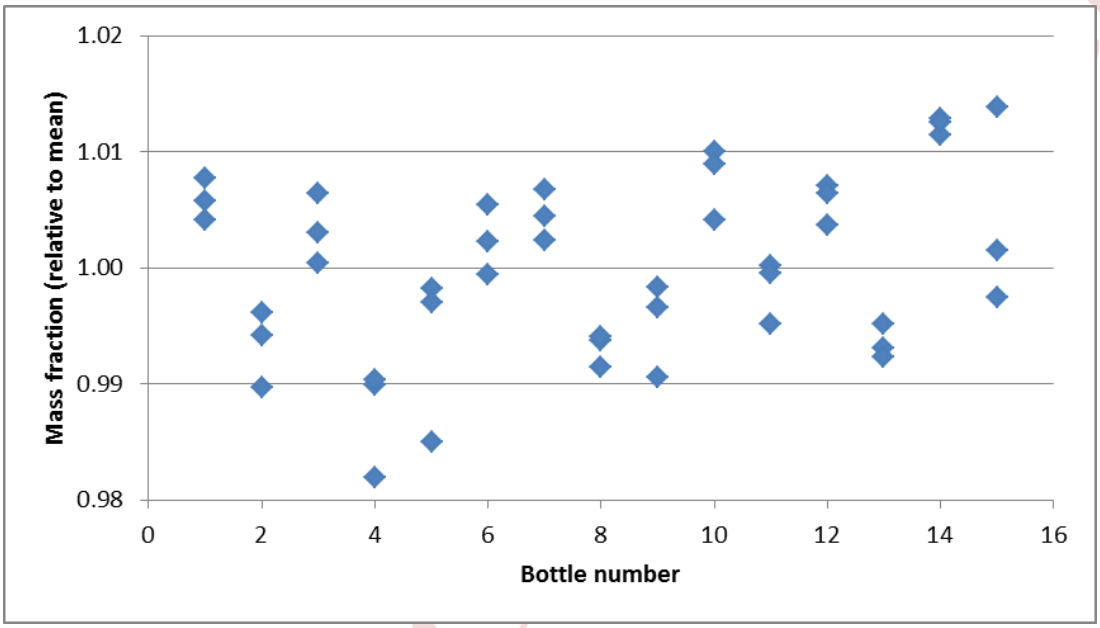
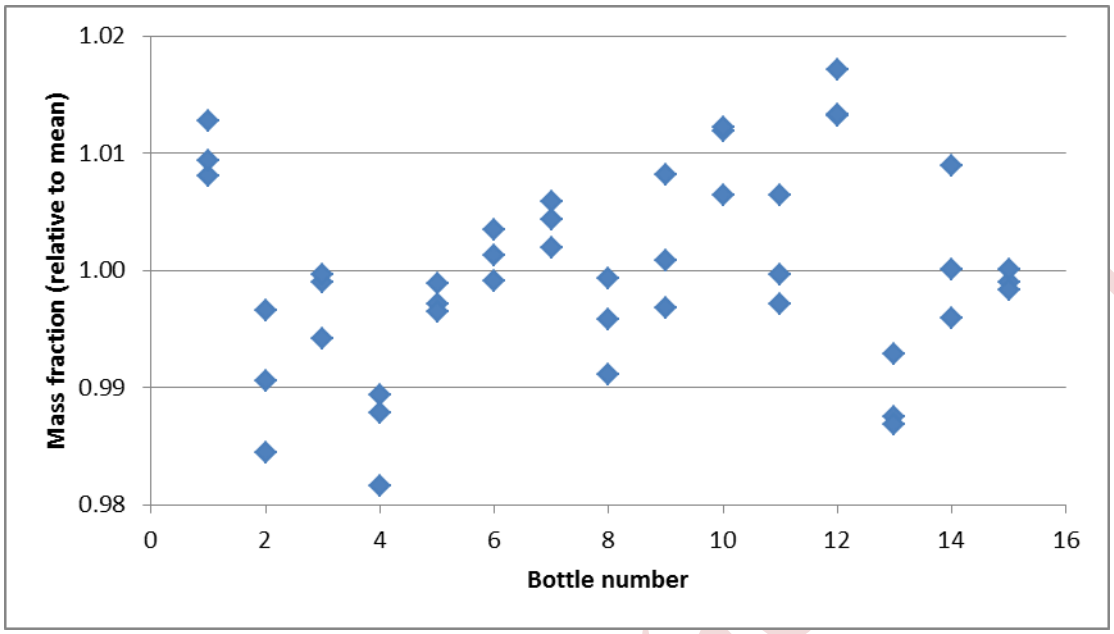


Figure 3. Homogeneity evaluation for Phenylalanine



Stability Assessment of Study Material

Short term stability assessment of 2 units in duplicate at four storage conditions, -80°C, –20°C, 4°C and 18°C. Short term stability was assessed at T0, 1 week, 2 weeks and 4 weeks (non-isochronously). Results for Leucine and Phenylalanine are shown in Figures 4 and 5 respectively, some instability is observed at 4°C and 18°C, but at -20°C and 80°C no significant change is observed. Long term stability (3 months and 6 months) results at -80°C are shown in Figures 6 and 7. No significant change in concentration is found over the time period.

Figure 4 Short term stability evaluation for Leucine

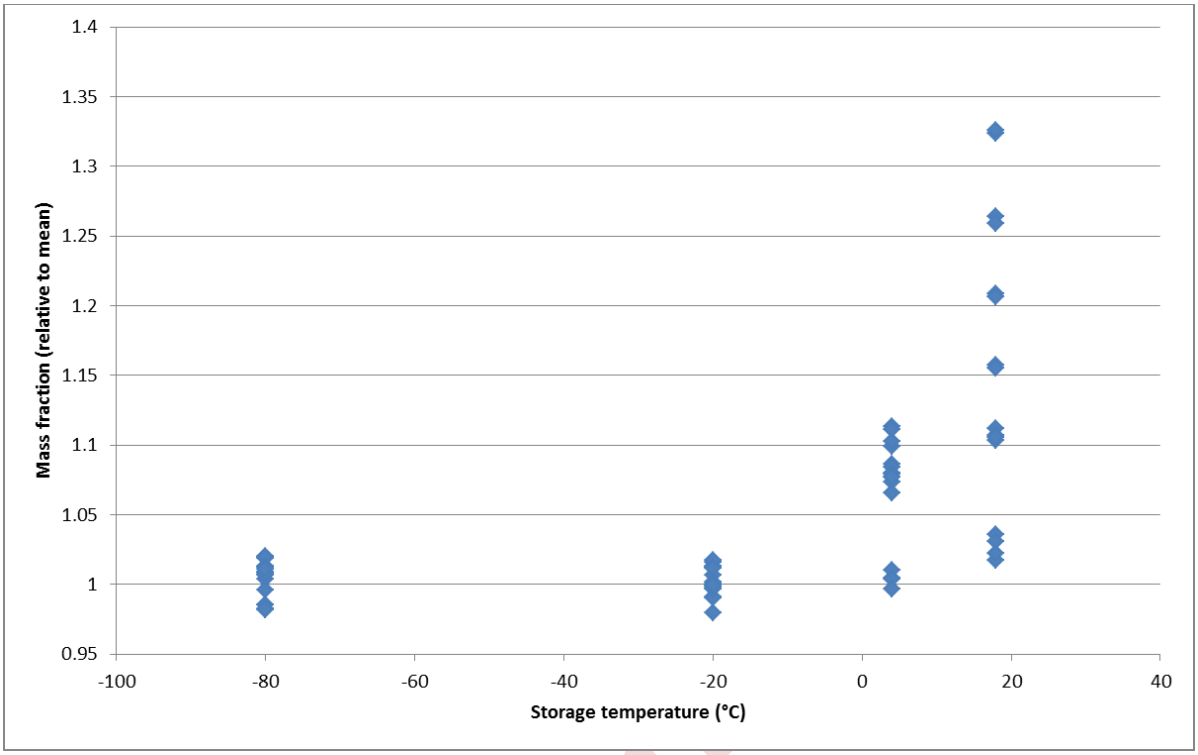


Figure 5 Short term stability evaluation for Phenylalanine

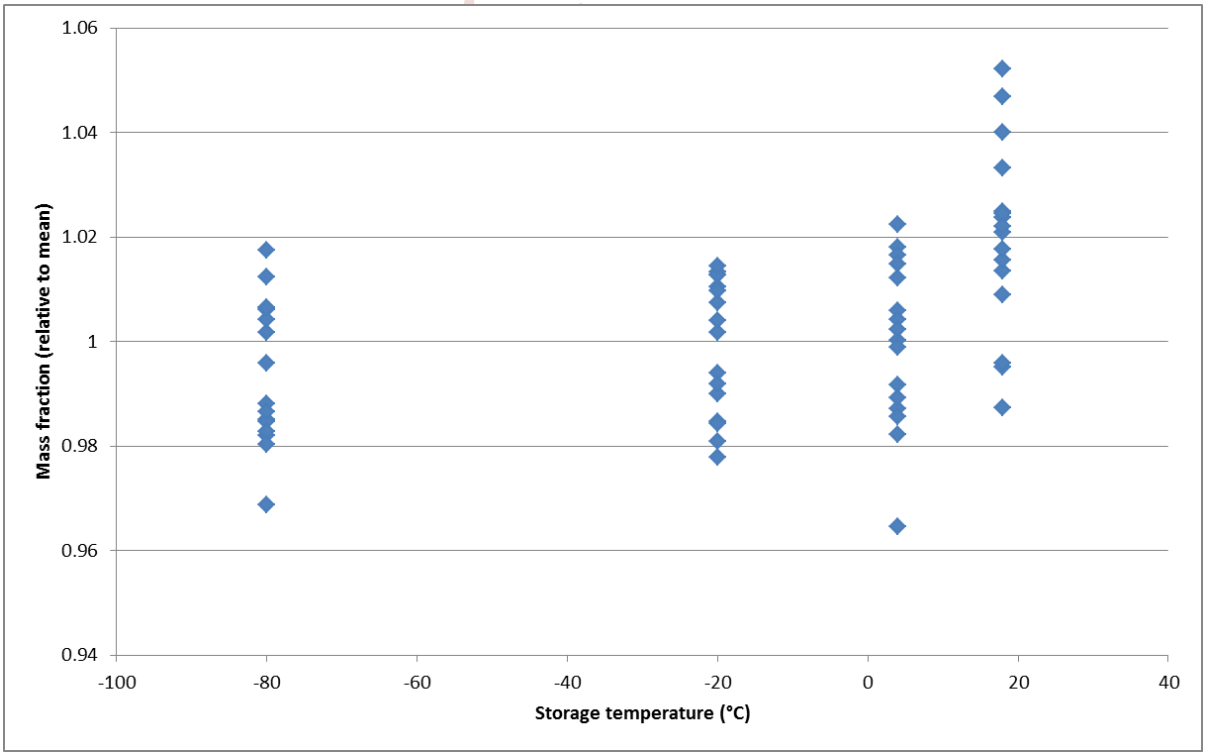


Figure 6 Long term stability evaluation for Leucine at -80°C

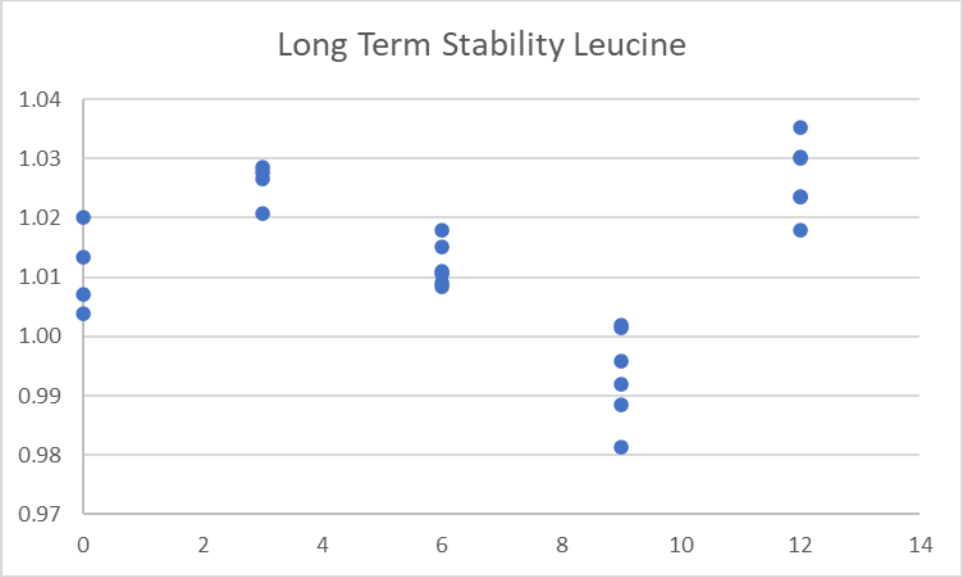
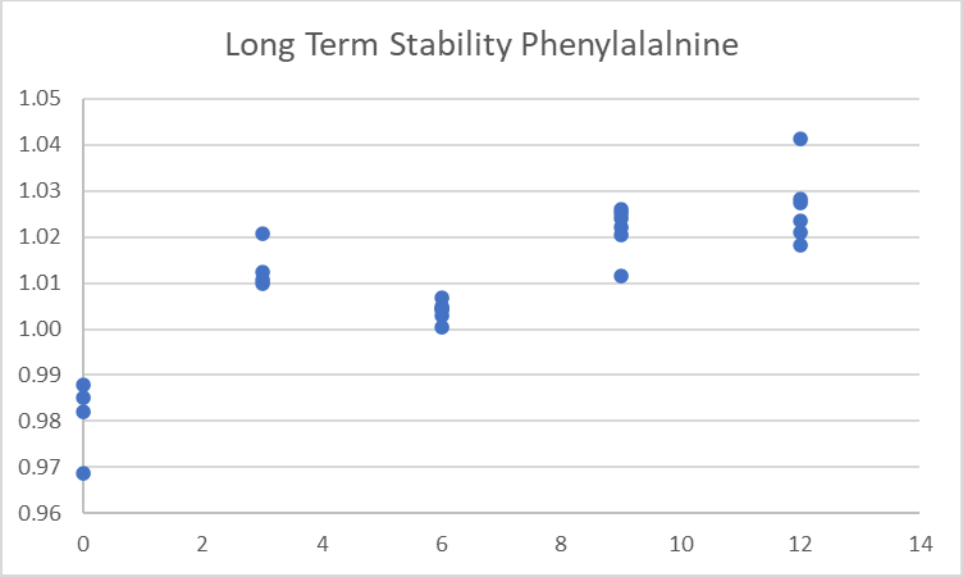


Figure 7 Long term stability evaluation for Phenylalanine at -80°C



As a final check, the long-term stability (LTS) data was analysed as a homogeneity data set for comparison, Table 3 shows the data from this analysis in comparison to the original homogeneity data set.

Table 3. Results of the Long-Term Stability data analysed as homogeneity data in comparison to the original homogeneity assessment data for free DL-leucine and DL-phenylalanine in frozen human plasma

Analyte	*Study*	*Between-unit SD*	*Within-unit SD*
Leucine	Homogeneity	0.56%	0.39%
	LTS as homogeneity	0.69%	0.34%
Phenylalanine	Homogeneity	0.53%	0.39%
	LTS as homogeneity	0.76%	0.44%

The data shows a slightly higher variance but this can be explained by the use of fresh analytical standards for each of the long term stability runs.

INSTRUCTIONS AND SAMPLE DISTRIBUTION

Samples will be shipped on dry ice and should be stored at -20°C until analysis, individual units are single use at point of measurement.

RESULTS

Participants will be requested to report a single estimate of the mass fraction in units of mg/kg for both DL-Leucine and DL-Phenylalanine. The reported mass fraction will be the overall mean from measurements from three separate units, reporting will include the values for the individual units in addition to the overall mean.

In addition to the quantitative results, participants will be instructed to describe their analytical methods, approach to uncertainty estimation, and the Core Competencies they felt were demonstrated in this study.

Available Calibration Materials

Participants may establish the metrological traceability of their results using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they determined the purity. Table 4 lists the CRMs that are available for use for this study.

Table 4: Certified Reference Materials Available for Use

CRM	Provider	Analyte	Mass Fraction[a] Delivered, mg/g	Mass Fraction[a] Source Material, mg/g	In-house Purity Methods Used to Value-Assign Source Material
CRM 6012-a	NMIJ	Leu	N/A	999 ± 2	Titration, LC-MS, TGA, Karl Fischer
CRM 6014-a	NMIJ	Phe	N/A	999 ± 2	Titration, LC-MS, TGA, Karl Fischer
CRM 2389a	NIST	Leu Phe	0.319 ± 0.014 0.421 ± 0.014	N/A	Gravimetric and LC-MS/MS
HRM 1008A	HSA	Leu	N/A	996.9 ± 3.3	LC-MS, TGA, Karl Fischer
HRM 1014A	HSA	Phe	N/A	997.5 ± 3.6	LC-MS, TGA, Karl Fischer

a Stated as Value ± U_{95}(Value)

Stable isotope internal standards for both leucine and phenylalanine are widely available.

USE OF CCQM-K159 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K159 will demonstrate measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Core Competency Statements and CMC support

Details of potential core competencies are listed in Appendix B.

APPENDIX C: Registration Form

CCQM - K159 / P202

Amino Acids in Plasma

Registration Form

My Institute/Laboratory would like to participate in Amino Acids in Plasma:

☐ Key Comparison K159 ☐ Pilot Study P202

Name of Institute	:	_____
Name of Department/ Laboratory	:	_____
Name of Contact Person	:	_____
Email Address	:	
Telephone Number	:	_____
Shipping Address	:	_____
		_____
Date	:	_____

Do you require a permit/licence to receive samples?

☐ No ☐ Yes (If yes, please send further details)

Please note that any import taxes or charges imposed on the comparison samples during transportation shall be borne by the participating laboratory.

The samples will be shipped on dry ice.

Please fill out this form using the Fill & Sign function in PDF and save it. Return the completed form to Chris Hopley and Tabatha Hambidge by **31/1/2019**.

If you do not receive an acknowledgement from us within 4 working days, please send us an email.

APPENDIX D: Reporting Form

Report of Results
CCQM-K159 and CCQM-P202
Free Amino Acids in Plasma

Please complete electronically

Participating Laboratory Submission Information

Participating Institute	
Reporting Date	
Submitter	
Position	
E-Mail	

Plasma Results Leucine

Bottle Number			
Result (mg/kg)			
Overall Mean (mg/kg)			
Combined Standard Uncertainty (mg/kg)			
Coverage Factor, k (95% confidence interval)			
Expanded uncertainty at 95% confidence interval (mg/kg)			

Plasma Results Phenylalanine

Bottle Number			
Result (mg/kg)			
Overall Mean (mg/kg)			
Combined Standard Uncertainty (mg/kg)			
Coverage Factor, k (95% confidence interval)			
Expanded uncertainty at 95% confidence interval (mg/kg)			

Technical information

Calibrants

	Leucine	Phenylalanine
Calibrant Source		
Purity (units)		
Expanded Uncertainty (units)		
Traceability		
Characterisation Technique(s)		
Purity Assay (if conducted)		

Internal Standards

	Leucine	Phenylalanine
Source		
Purity		
Labelling		

Measurement Procedure

	Leucine	Phenylalanine
Sample size used		
Sample preparation details (e.g. equilibration, extraction, clean-up procedures, derivatisation)		
Analysis Method (e.g. instruments/conditions, separation conditions, detection – wavelength, SRMs)		
Calibration strategy (e.g. IDMS, External calibration)		
Calibration method (e.g. single point, bracketing, multi-level)		
Calibration equation		

Uncertainty Budget

	Leucine	Phenylalanine
List and describe components of the uncertainty budget and contribution		

Additional Information

	Leucine	Phenylalanine
Any other relevant additional information		

Please return results via email to

Gill Holcombe, Head of Reference Material Production, LGC,

Gill.Holcombe@lgcgroup.com

APPENDIX E: Core Competency Tables

CCQM OAWG: Competency Template for Analyte(s) in Matrix

CCQM-K159	*NMIA*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *National Measurement Institute, Australia (NMIA)*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		Leucine: NMIJ CRM 6012-a Phenylalanine: NMIJ CRM 6014-a
Identity verification of analyte(s) in calibration material.	N/A	N/A
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	N/A
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	N/A
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*Retention times on three UHPLC columns* *LC-MSMS MRM ratios of four MRMs each for two chromatographic conditions*
Extraction of analyte(s) of interest from matrix	ü	*Protein precipitation with acetonitrile.*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	û	*N/A*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	ü	*Derivatisation with dansyl chloride*
Analytical system	ü	*2D-LC-MSMS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*Exact matching double isotope dilution mass spectrometry using a single point calibration with bracketing.*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*Two complimentary 2D-LC separations were used to verify lack of interferences.* *NIST 1950 CRM used as quality control*
Other	û	*N/A*

CCQM-K159	NMIJ/AIST	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	□,□, or N/A	Specific Information as Provided by NMIJ/AIST
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		CRMs were used for calibrant, Leu: NMIJ CRM 6012-a Phe: NMIJ CRM 6014-a
Identity verification of analyte(s) in calibration material. For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s). For calibrants which are a calibration solution: Value-assignment method(s).		*Indicate method(s) you used to identify analyte(s)* *Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)* *Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample	□	We confirmed the retention time, and roughly checked spike and recovery using calibrant.
Extraction of analyte(s) of interest from matrix	□	Protein precipitation by adding same volume of acetonitrile.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	**N/A**	*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	□	The analyte was derivatized with *N*-Butylnicotinic acid *N*-hydroxysuccinimide ester.
Analytical system	□	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	□	quantification mode, IDMS calibration mode, 5-point calibration curve
Verification method(s) for value-assignment of analyte(s) in sample (if used)	□	NIST SRM 1950 was used for confirmation of the validity.
Other		

CCQM-K159	*HSA*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, plasma and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *HSA*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	Ö	*High purity L-leucine and L-phenylalanine from HSA were used as the calibrants.*
Identity verification of analyte(s) in calibration material.	Ö	*LC-MS/MS was used to verify the $[M+H]^+$ ion and the corresponding daughter ions.*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	*L-leucine and L-phenylalanine CRMs certified using mass balance method were used as calibrants.*
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	Ö	*The analytes in the samples were identified against pure L-leucine and L-phenylalanine CRMs (HRM-1008A and HRM-1014A)) by comparing their MRM transitions and retention times on the LC-MS/MS.*
Extraction of analyte(s) of interest from matrix	N/A	
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	Ö	*Protein precipitation was used for clean-up. The details are as follows:* *After spiking the isotope labelled internal standard solution, the sample was vortexed, and allowed to equilibrate at ambient temperature for 2 h. Methanol (4-folds of aqueous volume) was then added for protein precipitation. The sample was vortexed vigorously and centrifuged for 10 min at 14000 g. The supernatant was filtered through 0.22 µm syringe filter. For LC-MS/MS analysis, the filtrate was diluted to approximately 100 ng/g with acetonitrile.*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	Ö	*One-step derivatisation was used for GC-MS measurement. The details are as follows:* *After centrifugation and filtration through 0.22 µm syringe filter, 50 µL of the filtrate 50 µL of pyridine and 50 µL of N-tert-Butyldimethylsilyl-N-methyl-trifuoroacetamide were added. The solution was vortexed vigorously for 1 min and was heated at 60 ^oC for 1 hour. The solution was then centrifuged at 4000 g for 5 min, and the supernatant was taken for GC-MS analysis.*
Analytical system	Ö	*LC-MS/MS and GC-MS were used. The averages of results obtained from LC-MS/MS and GC-MS were reported.*
Calibration approach for value-assignment of analyte(s) in matrix	Ö	*Four-point calibration curve IDMS method was used.*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	Ö	*Human plasma CRM for leucine and phenylalanine from NIST (SRM 1950) was used as quality control materials, which was measured in parallel with the comparison samples. The obtained values agreed well within the uncertainties of the reference values of the CRM. Human plasma CRMs for*

		leucine and phenylalanine from KRISS (111-01-019) (two concentration levels) were also measured to further verify our IDMS method. The obtained values were also found to be within the uncertainties of reference values of the CRMs.
Other	N/A	

CCQM-K159	*INMETRO*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	,û, or N/A	Specific Information as Provided by *INMETRO*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	ü	*We used the following highly-pure substance as calibrants: L-Leucine, L8000 (sigma-aldrich, St. Louis, USA) and L-Phenilalanine, P2126 (sigma-aldrich, St. Louis, USA.*
Identity verification of analyte(s) in calibration material.	ü	*The identity verification of the analyte was performed by ^{1}H NMR*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	ü	*Purity assessment method was ^{1}H NMR for both analytes.*
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	*Not applicable*
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*Analytes were identified in the sample by its retention time and by the use of an additional MS/MS transition for confirmation.*
Extraction of analyte(s) of interest from matrix	ü	*Sample preparations prior to LC-MS/MS analysis was conducted by the following steps: Protein precipitation using acetonitrile, evaporation (N_2 stream) to the dryness, reconstitution with 0.1 mol/L HCl solution.*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	ü	*Protein precipitation was used to cleanup the sample.*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	*Not applicable – no transformation was performed.*
Analytical system	ü	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*a) quantification mode used was IDMS* *b) calibration mode used was 5-point calibration curve*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*A different calibrant was used to confirm the value-assignment of analytes in the sample. The reported results were obtained from high-purity substances (sigma-aldrich) used as calibrants (identity verification and purity assessment performed by ^{1}H NMR). The results were compared to that obtained by the use of NIST SRM 2389a (Amino Acids in 0.1 mol/L Hydrochloric Acid) as calibrant.*

Other	ü	*NIST SRM 1950 (metabolites in human plasma) was used as control sample in order to check the measurement accuracy.*

CCQM-K159	*LGC*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *LGC*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*NMIJ pure materials* *L-Phenylalanine CRM 6014-a and L-Leucine CRM 6012-a*
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*2 MRM transitions, retention time, peak shape*
Extraction of analyte(s) of interest from matrix	ü	*Protein precipitation - 250µl plasma sample with 1 ml acetonitrile*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	N/A	
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	ü	*Derivatization of dried sample with 100µl MTBSTFA + 1% TBDMCS*
Analytical system	ü	*GC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*Bracketed single point double exact matching IDMS.*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*NIST SRM 1950 - Metabolites in Frozen Human Plasma*
Other	N/A	

CCQM-K159	*EXHM*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow> -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *EXHM*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	ü	*in house leucine and phenylalanine calibrant and calibration solution.*
Identity verification of analyte(s) in calibration material.	ü	*qNMR, ID- LC-MS/MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	ü	*qNMR against NMIJ CRM 4601*
For calibrants which are a calibration solution: Value-assignment method(s).	ü	*gravimetrically prepared calibration solutions assessed by LC-IDMS against NIST SRM 2389a*
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*Retention time, MRMs, mass spec ion ratios*
Extraction of analyte(s) of interest from matrix	ü	*After the addition of the internal standard solution, the analytes were extracted in cold methanol/ethanol by vortexing and simultaneous protein precipitation.*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	ü	*Centrifugation, evaporation, reconstitution and 20-fold dilution*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	ü	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*IDMS, exact matching* *matrix matched, single-point calibration,*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*- matrix-matched calibration against UME CRM 1314* *- standard additions*
Other	N/A	.

CCQM-K159	*KRISS*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *KRISS*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*High purity CRM of L-leucine (6012-a) and L-phenylalanine (6014-a), which of purities were certified by NMIJ.*
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	*Certified values assigned by NMIJ was used*
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*Retention time and mass spec ion ratios by ID-LC-MS/MS*
Extraction of analyte(s) of interest from matrix	ü	*Same amount of isotope solution (in DW) was added to plasma, then, gently mixed for 1 hour on a shaker.*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	ü	*Isotope-added samples were deproteinized with 15 %(v/v) 5-sulfosalicylic acid. After centrifuged, supernatants were filtered (0.2 μm), and diluted ten times with distilled water before applying LC-MS system*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	ü	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*a) Quantification mode : IDMS* *b) Calibration mode: exact-matched single-point calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*Analytical method was verified using KRISS CRMs 111-01-019 (amino acids in plasma)*
Other	N/A	.

CCQM-K159	*NIMT*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *National Institute of Metrology (Thailand) NIMT*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Highly-pure substances CRM, NMIJ CRM 6012-a L-Leucine and NMIJ CRM 6014-a L-Phenylalanine were used*
Identity verification of analyte(s) in calibration material.	ü	*LC-MS/MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	*The analytes in the samples were identified against NMIJ CRM 6012-a L-Leucine and NMIJ CRM 6014-a L-Phenylalanine standards by comparing their retention times, MS/MS spectra of both analytes*
Extraction of analyte(s) of interest from matrix	ü	*Protein precipitation using an equal amount of 30% 5-sulfosalicylic acid and centrifugation at 14000 rpm for 10 min*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	N/A	
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	ü	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	ü	*a) Exact-match IDMS* *b) Single-point bracketing calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	ü	*Spiked recovery*
Other	N/A	

CCQM-K159	*PTB*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *PTB*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	ü	Pure material from NMIJ: CRM 6012-a L-Leucine Pure material from NRC: APHE-1 L-Phenylalanine
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	ü	Retention time, mass spec ion ratios
Extraction of analyte(s) of interest from matrix	ü	Liquid/liquid
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	ü	Protein precipitation
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	ü	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix		a) IDMS b) single-point calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	
Other	N/A	

CCQM-K159	*TUBITAK UME*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *TUBITAK UME*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	□	*Highly pure substance,* *Leucine- Acros Organics, Cat no: 17213* *Phenylalanine- Acros Organics, Cat no: 13031*
Identity verification of analyte(s) in calibration material.	□	*IDMS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	□	*qNMR*
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	-
Sample Analysis Competencies		
Identification of analyte(s) in sample	□	*Ion ratios*
Extraction of analyte(s) of interest from matrix	□	*Liquid/liquid extraction*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	û	-
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	□	*Derivatization with propyl chloroformate*
Analytical system	□	*LC-HRMS*
Calibration approach for value-assignment of analyte(s) in matrix	□	*a) IDMS* *b) Calibration curve (5 point for Leucine; 6 point for Phenylalanine)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	-
Other	N/A	-

CCQM-K159	*VNIIM*	Free amino acids in plasma

Scope of Measurement: Successful participation in CCQM-K159 will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 50 g/mol to 500 g/mol, having high to mid-polarity pKow > -4, in mass fraction range from 1 mg/kg to 1000 mg/kg in a biological matrix such as human plasma, serum and urine.

Competency	ü,û, or N/A	Specific Information as Provided by *VNIIM*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	√	Commercially available highly-pure substances from Sigma-Aldrich Leucine # 61819 (lot#BCBV1129) Phenylalanine # 78019 (lot#BCBV5213)
Identity verification of analyte(s) in calibration material.	√	Retention time, MRM
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	√	The purity of highly-pure substances was determined in-house by mass balance approach. Structurally related organics: LC/MS, LC/LS, LC/DAD, GC/MS Moisture: Karl Fisher Titration VOC: GC/FID, GC/MS Non-volatiles: ICP/MS; TGA
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	Retention time, MRM
Extraction of analyte(s) of interest from matrix	√	Protein precipitation by organic solvent (methanol), centrifugation
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	Protein precipitation by organic solvent (methanol), centrifugation
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system	√	HPLC-MS/MS Agilent 6460 Triple Quad
Calibration approach for value-assignment of analyte(s) in matrix	√	IDMS Single point calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	√	Verification by using Matrix Reference Material UME CRM 1314 Verification by using SRM NIST 2389a
Other	N/A	*Indicate any other competencies demonstrated.*

APPENDIX F: Summary of Participants' Analytical Information

The following Tables summarize the detailed information about the analytical procedures each participant provided in their "Analytical Information" worksheets. The presentation of the information in many entries has been consolidated and standardized.

The participant's measurement uncertainty statements are provided verbatim in Appendix G.

Table F-1: Summary of Sample Size and Extraction for CCQM-K159

NMI/DI	Approximate sample size used (g)	Extraction
NMIA	0.200 uL 0.203 g	50 µL of a mixed leucine/phenylalanine internal standard solution was gravimetrically added to 200 µL of sample in a 2 mL Eppendorf tube and vortexed to mix. 750 µL of acetonitrile was added and the sample vortexed for 30 s and allowed to stand for 30 s before centrifugation at 14,000 rpm for 5 minutes. The resulting extract was decanted and a 20 µL aliquot was placed in a 1 mL glass vial. 100 µL of dansyl chloride solution (2 mg/mL in acetone) and 100 µL of 0.2 M sodium carbonate solution were added and the vial was capped and vortexed for 20 s then left to stand for 1 hour at room temperature protected from light. 100 µL of 0.2% formic acid in 1:1 acetonitrile:water was then added and the vial vortexed for 30 s. The derivatised extract was then analysed by LC-MSMS.
NMIJ	0.252 g 0.253 g 0.256 g	Isotopically labelled amino acids (Leu and Phe) were spiked into plasma sample. The mixture was equilibrated by gently shaking over night at 10 °C. The same volume (500 µL) of acetonitrile was added to the equilibrated mixture, and centrifuged. The supernatant was dried and reconstituted with borate buffer. The supernatant after centrifugation was derivatized with *N*-Butylnicotinic acid *N*-hydroxysuccinimide ester.
HSA	0.250 µL	The sample (250 µL) was weighed into a 2 mL plastic centrifuge tube, and was spiked with an appropriate amount of the internal standard. The mixture was vortexed vigorously for 1 min, and was allowed to equilibrate at room temperature for 2 hours. Methanol (4-folds of total aqueous solution) was then added to precipitate the protein. The mixture was vortexed for 1 min before being centrifuged at 14,000 g for 10 min. The mixture was then filtered through 0.22 µm syringe filter. The obtained clear solution was diluted with water to about 100 ng/g for LC-MS/MS analysis. For GC-MS analysis, an appropriate amount of the obtained clear solution was dried under nitrogen at 50 °C before 50 µL of pyridine and 50 µL of N-tert-butyldimethylsilyl-N-methyl-trifluoroacetamide were added. The solution was vortexed vigorously for 1 min and was heated at 60 °C for 1 hour. The solution was then centrifuged at 4,000 g for 5 min, and the supernatant was taken for GC-MS analysis, the concentration was about 4 µg/g.

INMETRO	0.300 g	Samples were spiked with 0.5 g of IS solution, at 0.036 mg/g, in acetonitrile. After that, 1 g of acetonitrile was added and the mixture was vortex mixed for 10 s, and centrifuged (5000 rpm, 10 min, 4 °C). The liquid phase was evaporated under nitrogen gas stream, at 40 °C until dryness. The residue was reconstituted in 0.1 mol/L HCl solution and filtered using a 0.22 µm PVDF syringe filter.
UME	0.250 µL	The analytes were derivatized with propyl chloroformate.
VNIIM	0.250 µL	Temperature stabilization – 1 hour Taking aliquot and adding Internal Standards (55 µl solution of 0.1M HCl) Adding 0,75 ml of cold (-18 °C) methanol Shaking by Vortex - 450 rpm, 20 minutes Centrifugation - 14600 rpm, 15 minutes Transferring the supernatant with a plastic Pasteur pipette into a 1.5 ml glass autosampler vial
NMIT	0.250 g	Protein precipitation- Dilute the plasma sample with water to 10-fold - Add 30 % 5-sulfosalicylic acid in an equal volume of plasma sample - incubate at 4°C for 10 min prior to centrifugation at 14,000 rpm for 10 min.
KRISS	0.500 g	Plasma and internal standard were gravimetrically mixed, then deproteinized with 15 %(v/v) 5-sulfosalicylic acid. After centrifuged, supernatants were filtered (0.2 µm), and diluted ten times with distilled water before applying LC-MS system
PTB	0.250 g	- Protein precipitation with cold Ethanol - Ultrafiltration with 3 kDa Ultra Centrifugal Filter - Extraction with tert-butyl methyl ether (TBME)
EXHM/GCSL-EIM	0.250 g	0.25 g of plasma sample (originally stored at -80°C and then -20°C), were thawed at room temperature for 30 min and vortexed for 30 sec. 0.25 g of 15N-labelled internal standard solution was added, vortexed and left for equilibration for 30 min at 4 °C. Then the sample was diluted with 1 mL of methanol (or ethanol) was vortexed for 30 sec and left at -20 °C for 30 min. The mix was allowed to thaw for 1 min, was vortexed for 30 sec and then centrifuged at 16000 x g for 15 min at 4 °C. The supernatant was evaporated to dryness at RT under vacuum. The residue was resuspended (by vortexing) in 1 mL of formic acid 0.1 %, then diluted 1:20 with acetonitrile:water 82:18 and analysed with LC-MS/MS.
LGC	0.250 g	1 hour equilibration of plasma with labelled analogue solution, protein precipitation with 1 mL MeCN, 100 µL supernatant dried before derivatisation with 100 µL MTBSTFA

Table F-2: Summary of Analytical Techniques for CCQM-K159

<table>
<tr><th>Institute</th><th>Analytical Technique</th><th>Chromatographic Column</th><th>Chromatographic and Mass Spectrometry Conditions</th><th>ion/MRM monitored</th></tr>
<tr>
<td>NMIA</td>
<td>2D LC-MS/MS</td>
<td>Method 1: D1 column - Waters CSH FluoroPhenyl 1.7 µm, 2.1 x 100 mm
D2 column - Restek Pinnacle DB BiPh 1.9 um, 2.1 x 100 mm
Method 2: D1 column - Waters CSH FluoroPhenyl 1.7 µm, 2.1 x 100 mm
D2 column -Waters BEH C18 1.7 µm, 2.1 x 100 mm</td>
<td>
Mobile Phases
A Water
B Acetonitrile
C Methanol
D 2% formic acid in water
Gradient
D1 Pumo / D2 Pump
Start (min) | Flow (mL/min) | %A | %B | %C | %D | Heartcut T | Flow (mL/min) | %A | %B | %C | %D
0 | 0.3 | 80 | 0 | 10 | 10 | | 0.3 | 0 | 0 | 99 | 1
1 | 0.3 | 62.2 | 0 | 27.8 | 10 | | 0.3 | 0 | 0 | 99 | 1
1.1 | 0.3 | 48.6 | 0 | 41.4 | 10 | | 0.3 | 87.5 | 0 | 10 | 2.5
7.92 | 0.15 | 48.5 | 0 | 41.5 | 10 | | 0.15 | 100 | 0 | 0 | 0
7.95 | 0.15 | 47.1 | 0 | 42.9 | 10 | T | 0.15 | 100 | 0 | 0 | 0
9.35 | 0.15 | 47 | 0 | 43 | 10 | | 0.15 | 100 | 0 | 0 | 0
9.38 | 0.3 | 46.8 | 0 | 43.2 | 10 | | 0.3 | 87.5 | 0 | 10 | 2.5
9.48 | 0.3 | 46.6 | 0 | 43.4 | 10 | | 0.3 | 51.9 | 0 | 45.6 | 2.5
9.58 | 0.3 | 46 | 0 | 44 | 10 | | 0.3 | 51.1 | 0 | 46.4 | 2.5
9.88 | 0.3 | 0 | 0 | 99 | 1 | | 0.3 | 29.7 | 0 | 67.8 | 2.5
16.98 | 0.3 | 80 | 0 | 10 | 10 | | 0.3 | 0 | 0 | 99 | 1
Gradient
D1 Pumo / D2 Pump
Start (min) | Flow (mL/min) | %A | %B | %C | %D | Heartcut T | Flow (mL/min) | %A | %B | %C | %D
0 | 0.3 | 80 | 0 | 10 | 10 | | 0.3 | 0 | 99 | 0 | 1
1 | 0.3 | 62.2 | 0 | 27.8 | 10 | | 0.3 | 0 | 99 | 0 | 1
1.1 | 0.3 | 48.6 | 0 | 41.4 | 10 | | 0.3 | 85 | 10 | 0 | 5
7.92 | 0.15 | 48.5 | 0 | 41.5 | 10 | | 0.15 | 100 | 0 | 0 | 0
7.95 | 0.15 | 47.1 | 0 | 42.9 | 10 | T | 0.15 | 100 | 0 | 0 | 0
9.35 | 0.15 | 47 | 0 | 43 | 10 | | 0.15 | 100 | 0 | 0 | 0
9.38 | 0.3 | 46.8 | 0 | 43.2 | 10 | | 0.3 | 85 | 10 | 0 | 5
9.48 | 0.3 | 46.6 | 0 | 43.4 | 10 | | 0.3 | 65 | 30 | 0 | 5
9.58 | 0.3 | 46 | 0 | 44 | 10 | | 0.3 | 64.1 | 30.9 | 0 | 5
9.88 | 0.3 | 0 | 0 | 99 | 1 | | 0.3 | 42.8 | 52.2 | 0 | 5
16.98 | 0.3 | 80 | 0 | 10 | 10 | | 0.3 | 0 | 99 | 0 | 1

Mass Spectrometer Settings (both methods)
Mode | Positive ESI
Spray Voltage | 3000 V
Vapouriser Temperatu | 400
Sheath Gas Pressure | 50
Aux Gas Pressure | 20
Capillary Temperature | 350
Collision Pressure | 1.5
Declustering Voltage | 5
Scan Width (m/z) | 0.01
Scan Time (s) | 0.04 each SRM
Q1 Peak Width | 0.7
Q2 Peak Width | 0.7
</td>
<td>
SRM Table
Analyte | Parent Mass | Product Mass | Collision Energy | S-Lens
Leucine | 365 | 115.1 | 56 | 86
365 | 156.1 | 40 | 86
365 | 157.1 | 25 | 86
365 | 170.1 | 20 | 86
13C6 15N Leucine | 372.1 | 115.1 | 61 | 86
372.1 | 156.1 | 41 | 86
372.1 | 157.1 | 28 | 86
372.1 | 170.1 | 20 | 86
Phenylalanine | 399.1 | 115.1 | 115.1 | 59
399.1 | 156.1 | 156.1 | 39
399.1 | 157.1 | 157.1 | 25
399.1 | 170.1 | 170.1 | 20
13C9 15N Phenylalanir | 409 | 115.1 | 115.1 | 56
409 | 156.1 | 156.1 | 39
409 | 157.1 | 157.1 | 25
409 | 170.1 | 170.1 | 22
</td>
</tr>
</table>

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored
NMIJ / AIST	LC-MS/MS	Develosil C30-UG-5, 5 µm, 2.0 mm i.d. ×250 mm (Nomura Chemical, Japan)	Solvent A: 0.05 %TFA, 0.5 %Formic acid/water, B: 0.05 % TFA, 0.5 % Formic acid/MeCN, 5 % B (0→5min) 5→20 %B (5→15min), 20→55 %B (15→25min) Flow rate 0.2 mL/min, Column temperature 40 ℃ MS/MS conditions: operated under selected reaction monitoring	Leu: 293.19 → 247.18, Phe: 327.17 → 281.17 13C615N-Leu: 300.20 → 253.20, 13C915N-Phe: 337.20 → 290.19
HSA	LC-MS/MS	ZORBAX ECLIPSE AAA, 4.6 x 75 mm, 3.5 µm	Mobile phase A: 0.1 % TFA in water. Mobile phase B: 0.1 % TFA in acetonitrile. Flow rate: 0.3 mL/min. Gradient: 10 – 60 % B. Injection volume: 10 µL	MRM transitions: 132/86 (quantifier) and 132/44 (qualifier) for leucine; 138/91 (quantifier) and 138/46 (qualifier) for 13C6-leucine. MRM transitions: 166/120 (quantifier) and 166/103 (qualifier) for phenylalanine; 172/126 (quantifier) and 172/109 (qualifier) for ring-13C6-phenylalanine.
HSA	GC-MS	DB5-MS, 15 m × 0.25 µm × 0.25 mm	Oven condition: 100 °C, 30 °C/min to 200 °C, 10 °C/min to 250 °C, 40 °C/min to 300 °C, post run 300 °C for 1 min.	Mass monitored: 200.2 (quantifier) and 274.2 (qualifier) for leucine; 205.2 (quantifier) and 279.2 (qualifier) for 13C6-leucine. Mass monitored: 234.2 (quantifier) and 336.2 (qualifier) for phenylalanine; 240.2 (quantifier) and 342.2 (qualifier) for ring-13C6-phenylalanine.

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored
INMETRO	HPLC-MS/MS	C18 column (150 mm x 4.6 mm x 4 µm)	NST (Nano Separation Technologies, SP, Brazil) Flow rate of 0.6 mL/min and gradient mode with solvent A (water with 20 mM ammonium formate buffer, at pH 3.0), and solvent B (acetonitrile-water, 90:10 v/v, with 20 mM ammonium formate buffer, at pH 3.0). The elution program was: initial to 5 min, 10 % B; 9 min, 80 % B; 12 min, 80 % B; 14 min, 10 % B; 20 min, 10 % B. The mass spectrometer was operated in positive ESI mode	m/z 132>86 (10 V collision energy) for quantification and m/z 132>43 (20 V collision energy) for confirmation. For the IS, the transitions monitored were m/z 135>89 (10 V collision energy) and m/z 135>46 (22 V collision energy). The ESI conditions were: Capillary voltage: 3.0 V, cone voltage: 20 V, m/z 166>120 (13 V collision energy) for quantification and m/z 166>103 (25 V collision energy) for confirmation. For the IS, the transitions monitored were m/z 171>125 (15 V collision energy) and m/z 171>106 (30 V collision energy). The ESI conditions were: Capillary voltage: 3.0 V, cone

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored
UME	Thermo Scientific Q Exactive, Orbitrap LC/MS system	Troyasil C18 3 µm, 250x2.1 mm	The flow rate of the mobile phase was 0.250 mL/min, and the column temperature was set to 40 °C. Injection volume was 2 µL. The run time: 22.0 min. The mobile phase was composed of (A: MeOH: H2O (1:1)(%0.1 formic acid) (1:1), B: MeOH (%0.1 formic acid) Retention time (min) Flow (mL/min) A % B % 0.00 0.250 62 38 0.00 0.250 62 38 1.00 0.250 62 38 12.00 0.250 35 65 12.01 0.250 5 95 14.00 0.250 5 95 14.01 0.250 62 38 20.00 0.250 62 38	

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored				
VNIIM	HPLC-MS/MS Agilent 6460 Triple Quad	YMC Hydrosphere C18 100*4,6 mm*3 µm	Eluent A: water + 0.1 % HFBA Eluent B: acetonitrile + 0.1 % HFBA Program: 0 min – 0 % B; 15 minutes – 15 % B; 17 minutes – 16 % B; 18 min – 0 % B% 25 min - 0% B. Eluent flow: 0,8 µl/min	**Compound Name**	**Leu**	**Leu C13**	**Phe**	**Phe C13**
				PrecursorIon	132,1	139,2	166,1	176,1
				ProductIon	86,2	92,1	120,1	129,1
				Dwell	200	200	200	200
				Fragmentor	88	104	80	90
				Collision Energy	9	9	13	13
				CellAccelerator Voltage	4	4	4	4
				Polarity	Positive	Positive	Positive	Positive

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored
NMIT	LC-MS/MS	Intrada Amino Acid, 3 µm, 150 × 3 mm.	Mobile Phase A: ACN/THF/25mM ammonium formate /formic acid, 9:75:16:0.3 (v/v/v/v) Mobile Phase B: ACN/ 100 mM Ammonium formate, 20:80 (v/v) Gradient elution: 0% B (0-2 min), 0-30 % B (2-6.5 min), 100% B (6.5-10 min), 100 %B (10-12 min), 0 %B (12-13min) Run time: 15 min Flow rate: 0.6 mL/min Column oven: 35 °C Injection volume: 1 µL API 4000 QTRAP, AB SCIEX Ionization: ESI positive mode	132.17 > 86.06 for leucine 139.15> 92.07 for Isotope labeled leucine 166.12 > 120.07 for Phenylalanine 176.20>129.12 for Isotope labeled phenylalanine
KRISS	LC-MS/MS	Capcell Pak ADME (2.1 X 150 mm, 2.7 µm)	Mobile phase : Isocratic elution of 5% TFA at 0.25 mL/min Injected amount : 3 µL Column temperature : 30 ℃ SCIEX qTOF 5600 ESI positive mode GS1 – 50 psi, GS2-50 psi, CUR - 30 psi, Temp. 500 ℃ DP – 80 eV,	<Leu> m/z 132.1 → 86.1 (unlabeled) m/z 139.1 → 92.1 (labeled) CE – 15 eV <Phe> m/z 166.1 → 120.1 (unlabeled) m/z 176.1 → 129.1 (labeled) CE – 17 eV

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions	ion/MRM monitored
PTB	LC-MS/MS	ZIC®-HILIC, 3,5 µm, 200Å, 150 x 2.1 mm SeQuant	4000 QTrap Sciex Scan Type: Q1 Selected ion monitoring Polarity: positive Chromatographic Conditions: Isocratic elution 20:80 (v/v) 5 mmol/l ammonium acetate / acetonitrile, Flow Rate: 0.1 ml/min Column Oven: 25°C Injection Volume: 2 µl	m/z: 132,1 Leu m/z: 138,1 Spike Leu m/z: 166,1 Phe m/z: 176,1 Spike Phe
EXHM/GCSL-EIM	LC-MS/MS	Sequant ZIC-HILIC (150 x 2.1 mm, 5 µm, 200 Å)	isocratic - 82.5 % acetonitrile / 17.5 % 20 mM acetic acid 10 mM ammonium acetate	detection-SRM: LEU (132 to 86q and 43) - 15N -LEU (133 to 87q and 43) PHE (166 to 120q and 103) - ^{15}N- PHE (167 to 121q and 103)
LGC	GC-MS/MS	Restek Rxi-5HT 30m x 0.25 mm, 0.25 µm	Split injection, GC temperature ramp separation MS/MS detection	MRM transitions: 302>274 and 200>88 MRM transitions: 336>308 and 336>204

Table F-3: Summary of Calibrants and Standards for CCQM-K159

Institute	Type of Calibration	Calibrants	Internal Standards
NMIA	Exact matching double IDMS, Single point bracketing	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6, 15N
NMIJ	IDMS, 5-point calibration	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6, 15N
HSA	IDMS, Multi-level (4-point calibration curve)	HRM-1014A 0.9975 ± 0.0033 HRM-1008A 0.9969 ± 0.0036	Ring-13C6-L-Phenylalanine 13C6-L-Leucine
INMETRO	IDMS, 5-level internal calibration curve, using labelled analogue compounds as internal standards	L-Phenylalanine, P2126, Sigma-Aldrich, purity assigned by qNMR at INMETRO 99.902 ± 0.095 g/100g L-Leucine, L8000, Sigma-Aldrich, purity assigned by qNMR at INMETRO 99.86 ± 0.1 g/100g	L-Phenyl-d5-alanine L-Leucine-5,5,5-d3
UME	Calibration curve (5 point leucine, 6 point phenylalanine)	L-Phenylalanine, Agros Organics, purity assigned by qNMR at UME 99.70 % ± 0.23 % L-Leucine, Agros Organics, purity assigned by qNMR at UME, 99.63 % ± 0.23 %	Leucine 1-13C Phenylalanine Ring-D5

Institute	Type of Calibration	Calibrants	Internal Standards
VNIIM	IDMS Single point calibration by 3 calibration solutions	L-Phenylalanine, Sigma 78019, purity assigned by mass balance at VNIIM 999.62 ± 0.3 mg/g L-Leucine, Sigma 61819, purity assigned by mass balance at VNIIM, 997.5 ± 0.3 mg/g	Phenylalanine 13C9, 15N Leucine 13C6, 15N
NMIT	IDMS Single point calibration	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6, 15N
KRISS	IDMS exact-matched single-point calibration	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6, 15N
PTB	IDMS Single point calibration	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6
EXHM/GCSL-EIM	IDMS single point at exact matching Also verified by additional experiments a) matrix matched standards at exact matching (with the use of UME CRM 1314). standard additions	L-Phenylalanine, purity assigned by qNMR at EXHM, 999.1 ± 2.2 mg/g L-Leucine, purity assigned by qNMR at EXHM, 997.7 ± 2.1 mg/g	Phenylalanine 15N Leucine 15N
LGC	Double Exact Matching Isotope Dilution Mass Spectrometry (DEM-IDMS)	CRM 6014-a 0.999 ± 0.002 CRM 6012-a 0.999 ± 0.002	Phenylalanine 13C9, 15N Leucine 13C6, 15N

Table F-4 Assessment and Verification Methods for CCQM-K159

Institute	Purity Assessment	Result Verification
INMETRO	1H qNMR	Use of CRM (Maleic acid, INMETRO, 8792.0001) as internal standard in qNMR purity analysis, associated with the gravimetric preparation of the solutions (primary techniques) for the NMR analysis.
UME	qNMR	
VNIIM	Purity of high purity chemicals was determined in-house by mass balance approach LC/MS, LC/LS, GC/MS, LC/DAD, GC/FID, ICP/MS, TGA, KF titration	
EXHM	Purity was determined with the 1H-qNMR method against NMIJ CRM 4601-a	The gravimetrically assigned value of the calibration solutions was assessed by LC-IDMS against NIST SRM 2389a

Table F-5: Additional Comments for CCQM-K159

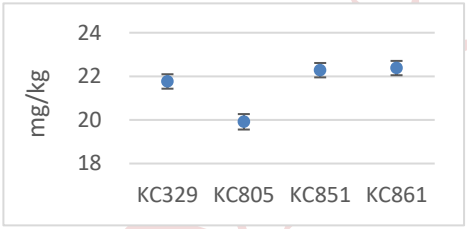
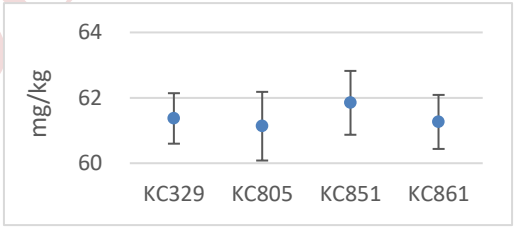
<table>
<tr><th>Institute</th><th colspan="2">Additional Comments</th></tr>
<tr><td>NMIA</td><td>
<table>
<tr><th>Leucine</th><th>Phenylalanine</th></tr>
<tr><td>Bottle 240 also analysed but not included as it is an outlier (20.91 mg/kg).

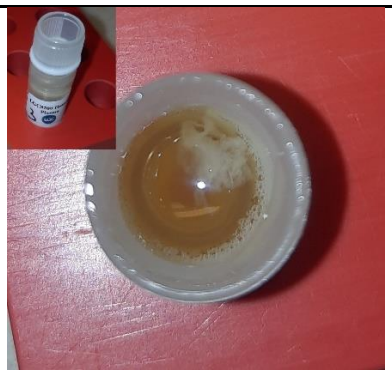
Bottle 787 analysed to determine indicative values only (IDMS with multipoint calibration curve), with a mass fraction of 18.9 mg/kg

NIST 1950 used as quality control</td><td>Bottle 240 also analysed and included in the overall mean and estimate of uncertainty, with a mass fraction of 61.7 mg/kg.

Bottle 787 analysed to determine indicative values only (IDMS with multipoint calibration curve).

NIST 1950 used as quality control</td></tr>
</table>
</td></tr>
<tr><td>NMIJ/AIST</td><td colspan="2">N/A</td></tr>
<tr><td>HSA</td><td>
<table>
<tr><th>Leucine</th><th>Phenylalanine</th></tr>
<tr><td>Human plasma CRM for leucine from NIST (SRM 1950) was used as quality control materials, which was measured in parallel with the comparison samples. The obtained value agreed well within the uncertainty of the reference values of the CRM. Human plasma CRMs for leucine from KRISS (111-01-019) (two concentration levels) were also measured to further verify our IDMS method. The obtained values were also found to be within the uncertainties of reference values of the CRMs.</td><td>Human plasma CRM for phenylalanine from NIST (SRM 1950) were used as quality control materials, which was measured in parallel with the comparison samples. The obtained value agreed well within the uncertainty of the reference values of the CRM. Human plasma CRMs for phenylalanine from KRISS (111-01-019) (two concentration levels) were also measured to further verify our IDMS method. The obtained values were also found to be within the</td></tr>
</table>
</td></tr>
</table>

<table>
<tr><td></td><td><table><tr><td></td><td>uncertainties of reference values of the CRMs.</td></tr></table></td></tr>
<tr><td>INMETRO</td><td>N/A</td></tr>
<tr><td>KRISS</td><td>
<table>
<tr><th>Leucine</th><th>Phenylalanine</th></tr>
<tr><td>The leucine value in #805 was excluded, due to lower measured value (19.91 mg/kg) that cause U_{exp} up to 10%. (*Sub-sampling result was 19.67 mg/kg). It seems necessary to reconfirm the homogeneity. The results were estimated with KC 329,851, and 861 samples, except #805.
 
</td><td>Measured value of phenylalanine was homogenous between all vials. Same as Leucine, the results were estimated from KC 329,851, and 861 samples.
 
</td></tr>
<tr><td colspan="2">In the vials of #805 and #851, a yellow-white clot was found. It floated in plasma and did not dissolved by vortex mixing. Samples were taken by avoiding the clot. However, we do not conclude that this clot affected the measurement results.
  (The clot attached at vial cap in #851)</td></tr>
</table>
</td></tr>
<tr><td>PTB</td><td>
<table>
<tr><th>Leucine</th><th>Phenylalanine</th></tr>
<tr><td colspan="2">White / yellow jelly-like precipitate in each vial see picture.</td></tr>
</table>
</td></tr>
</table>

<table>
<tr><td></td><td>  </td><td></td></tr>
<tr><td>UME</td><td><table><tr><th>Leucine</th><th>Phenylalanine</th></tr><tr><td>Single point ID-MS method was also applied.
The results of Leu for 3 units are: 19.23 mg/kg, 19.17 mg/kg, 20.97 mg/kg.</td><td>Single point ID-MS method was also applied.
The average result of Phe for 3 units is: 57.63 mg/kg.</td></tr></table></td><td></td></tr>
<tr><td>VNIIM</td><td><table><tr><th>Leucine</th><th>Phenylalanine</th></tr><tr><td>Some kind of protein aggregation was found in the each of the Samples (please, see the figure on the right).
Aliquots were taken in such a way to avoid the taking of this aggregation.

The measurements of 2 Samples (#LGC8280/001/0874, #LGC8280/001/0621) were carried out by alternative way - Internal Standards were added to the whole Sample and after that the aliquots were taken and analyzed by the same procedure.
The results are:
Leucine – 24.2 mg/kg
Phenylalanine – 62.5 mg/kg</td><td>  </td></tr></table></td><td></td></tr>
</table>

APPENDIX G: Summary of Participants' Uncertainty Estimation Approaches

The following are text excerpts and/or pictures of the uncertainty-related information provided by the participants in the reporting form. Information is grouped by participant and presented in alphabetized acronym order.

Uncertainty Information from EXHM/GCSL-EIM

L-leucine

uncertainty component-Leucine	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	19,97	1,000	0,33	0,0165	0,3300	0,1089
mass fraction of L-leucine in the calibration solution, (mg/kg)	19,97	1,000	0,40	0,0200	0,4000	0,1600
recovery (%)	100,00	-0,200	3,50	0,0350	-0,6980	0,4871
mass of Leu-N_{15} solution added to sample blend, (g)	0,25000	79,880	0,00003	0,0001	0,0024	0,0000
mass of test material in sample blend, (g)	0,25000	-79,880	0,00003	0,0001	-0,0024	0,0000
mass of Leu solution added to calibration blend, (g)	0,25000	79,880	0,00003	0,0001	0,0024	0,0000
mass of Leu-N_{15} solution added to calibration blend, (g)	0,25000	-79,880	0,00003	0,0001	-0,0024	0,0000
measured peak area ratio of the selected ions in the sample blend	0,665	30,030	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	0,665	-30,030	estimation of method precision			
result (mg/kg)	19,97					
combined standard uncertainty (mg/kg)	0,87					
relative standard uncertainty (%)	4,35					
coverage factor	2,00					
expanded uncertainty (mg/kg)	1,74					

L-phenylalanine

uncertainty component - Phenylalanine	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	59,74	1,000	0,18	0,0030	0,1800	0,0324
mass fraction of L-phenylalanine in the calibration solution, (mg/kg)	59,74	1,000	0,83	0,0139	0,8315	0,6914
recovery (%)	100,00	-0,597	3,32	0,0332	-1,9834	3,9337
mass of Phe-N_{15} solution added to sample blend, (g)	0,25000	238,960	0,00003	0,0001	0,0072	0,0001
mass of test material in sample blend, (g)	0,25000	-238,960	0,00003	0,0001	-0,0072	0,0001
mass of Phe solution added to calibration blend, (g)	0,25000	238,960	0,00003	0,0001	0,0072	0,0001
mass of Phe-N_{15} solution added to calibration blend, (g)	0,25000	-238,960	0,00003	0,0001	-0,0072	0,0001
measured peak area ratio of the selected ions in the sample blend	0,896	66,674	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	0,896	-66,674	estimation of method precision			
result (mg/kg)	59,74					
combined standard uncertainty (mg/kg)	2,16					
relative standard uncertainty (%)	3,61					
coverage factor	2,00					
expanded uncertainty (mg/kg)	4,32					

Uncertainty Information from HSA

Leucine	Phenylalanine
Not applicable. As instructed by the coordinating institute, the overall mean and combined uncertainty for leucine were not reported.	1. Mass of plasma sample (Type B) Value: 0.2515 g Standard uncertainty: 0.00078 g Relative uncertainty: 0.031 % Sensitivity coefficient: 241.85 Contribution: 0.05 % 2. Mass of internal standard solution (Type B) Value: 0.0492 g Standard uncertainty: 0.00078 g Relative uncertainty: 0.158 % Sensitivity coefficient: 1236.32 Contribution: 1.35 % 3. Concentration of calibration solution (Type B) Value: 5337.9 mg/kg Standard uncertainty: 18.2 mg/kg Relative uncertainty: 0.340 % Sensitivity coefficient: 0.011 Contribution: 6.26 % 4. Linear regression of the calibration curve (Type A) Value: 1.0085 Standard uncertainty: 0.00698 Relative uncertainty: 0.692 % Sensitivity coefficient: 60.30 Contribution: 25.84 % 5. Method precision including both LC-MS/MS and GC-MS methods (Type A) Value: 60.81 mg/kg Standard uncertainty: 0.636 mg/kg Relative uncertainty: 1.05 % Sensitivity coefficient: 1 Contribution: 59.12 % 6. Variation between quantifying and qualifying ion pairs (Type A) Value: 60.82 mg/kg

	Standard uncertainty: 0.225 mg/kg Relative uncertainty: 0.370 % Sensitivity coefficient: 1 Contribution: 7.38 %

Uncertainty Information from INMETRO

Leucine			Phenylalanine		
Source	**Uncertainty (mg/kg)**	**Contribution (%)**	**Source**	**Uncertainty (mg/kg)**	**Contribution (%)**
Instrument repeatability	0.141	1.600	Instrument repeatability	0.593	20.116
Sample mass	0.005	0.002	Sample mass	0.014	0.011
Mass of IS solution added	0.003	0.001	Mass of IS solution added	0.008	0.004
Calibration curve	0.062	0.312	Calibration curve	0.561	17.994
Purity	0.011	0.010	Purity	0.029	0.048
Between sample variation	0.895	64.258	Between sample variation	0.402	9.232
Bias	0.649	33.818	Bias	0.959	52.595
Combined standard uncertainty	1.1	100	Combined standard uncertainty	1.3	100

Uncertainty Information from KRISS

Leucine	Phenylalanine

Category	Factor	Leu	Phe
Systematic u (u_{std})	Uncertainty of purity of primary reference material	0.20%	0.20%
	Uncertainty of gravimetric preparation for standard solutions	0.40%	0.53%
	Uncertainty of gravimetric mixing for calibration isotope standard mixtures	0.72%	0.72%
	Area ratio of native/isotope for the calibration standard mixture, observed by LC-MS	0.26%	0.60%
Random u (u_{sam})	Measurement of sample solutions including homogeneity (s^2/n)	0.86%	0.29%
u_{comb}	$\sqrt{(u_{std}^2 + u_{sam}^2)}$	1.24%	1.13%
v_{eff}	Welch-Satterthwaite formula	8	11
$k(>95\%)$	t-table	2.31	2.20
U_{exp}	k x u_{comb}	2.85%	2.49%

Uncertainty Information from LGC

Leucine	Phenylalanine
RSB/RCB (bracketed) – uncertainty associated with replicate instrument measurements – 95 % Wz – Uncertainty of the balances used to prepare calibration standards and purity of pure standards – 3.5 % mx, myc, mz and my – uncertainty of balance used to prepare blends – 1.5 %	RSB/RCB (bracketed) – uncertainty associated with replicate instrument measurements – 95 % Wz – Uncertainty of the balances used to prepare calibration standards and purity of pure standards – 3.5 % mx, myc, mz and my – uncertainty of balance used to prepare blends – 1.5 %

Uncertainty Information from NMIA

Leucine Estimation of Measurement Uncertainty

Equation $W_x = W_z . \frac{m_y}{m_x} . \frac{m_{zc}}{m_{yc}} . \frac{R'_b}{R'_{bc}} . P . F_{MRM} . F_{Bias}$

Uncertainty Components

Factor	Value	u(x)	u(x)/x	Veff	dW/dx	$(c.u(x))^2$	$(c.u(x))^4/Veff$
P	1	0.001048282	0.105%	2	0.01903326	3.98091E-10	7.92383E-20
mx	0.20324	4.08248E-05	0.020%	400	-0.093649183	1.46169E-11	5.34138E-25
my	0.04954	4.08248E-05	0.082%	400	0.384161066	2.45966E-10	1.51248E-22
mzc	0.04948	4.08248E-05	0.082%	400	0.384626857	2.46563E-10	1.51983E-22
myc	0.04989	4.08248E-05	0.082%	400	-0.381466279	2.42528E-10	1.47049E-22
Wz	0.080	0.000402312	0.504%	3.78	0.238246735	9.18711E-09	2.23416E-17
R'b	0.960	Considered as part of precision					
R'bc	0.969						
FMRM	1	0.004734505	0.473%	14	0.01903326	8.12036E-09	4.71002E-18
FBias	1	0.01	1.000%	20.00	0.01903326	3.62265E-08	6.5618E-17

Phenylalanine Estimation of Measurement Uncertainty

Equation $W_x = W_z . \frac{m_y}{m_x} . \frac{m_{zc}}{m_{yc}} . \frac{R'_b}{R'_{bc}} . P . F_{MRM} . F_{Bias}$

Uncertainty Components

Factor	Value	u(x)	u(x)/x	Veff	dW/dx	$(c.u(x))^2$	$(c.u(x))^4/Veff$
P	1	0.00900834	0.901%	3	0.061369841	3.05632E-07	3.11371E-14
mx	0.20324	4.08248E-05	0.020%	400	-0.301957494	1.51964E-10	5.77326E-23
my	0.04954	4.08248E-05	0.082%	400	1.238668708	2.55717E-09	1.63478E-20
mzc	0.04948	4.08248E-05	0.082%	400	1.24017058	2.56337E-09	1.64272E-20
myc	0.04989	4.08248E-05	0.082%	400	-1.22997978	2.52142E-09	1.58939E-20
Wz	0.249	0.000651276	0.262%	6.95	0.246740456	2.58232E-08	9.60063E-17
R'b	1.009	Considered as part of precision					
R'bc	1.000						
FMRM	1	0.004128526	0.413%	19	0.061369841	6.41948E-08	2.16894E-16
FBias	1	0.01	1.000%	20.00	0.061369841	3.76626E-07	7.09235E-15

Uncertainty Information from NMIJ / AIST

Leucine	Phenylalanine	
Uncertainty components (rel, %)	**Leu**	**Phe**
Precision between vials	0.649	0.000
Precision between measurements	0.280	0.491
Weighing of solution	0.088	0.069
Purity of amino acid standard	0.100	0.100
Calibration curve	0.232	0.141
Combined uncertainty (%)	0.755	0.525
Combined uncertainty (mg/kg)	0.15	0.32
Expanded uncertainty (mg/kg), *k*=2	0.30	0.64

Uncertainty Information from NMIT

Leucine

Wx = 19.28 mg//kg
u(x) = 0.44 mg//kg
u(x)/x = 2.26 %
Veff (total) = 95.938
k= 1.99 (@ 95 % level)
U(x) = 0.87
%U(x) = 4.48 %

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Method Precision	19.2826	0.201942	1.047 %
m_{zc}	0.04333	0.000083	0.1916 %
m_y	0.04578	0.000083	0.1813 %
m_{yc}	0.04662	0.000083	0.1781 %
m_x	0.09872	0.000083	0.0841 %
w_z	19.8043	0.235027	1.1867 %
R'b	0.9396	0.008642	0.9198 %
R'bc	0.9386	0.006780	0.7224 %
Sample preparation effect	1.000	0.0100	1.000 %
Recovery	0.998	0.003218	0.322 %

Phenylalanine

Wx = 60.3 mg//kg
u(x) = 1.2 mg//kg
u(x)/x = 1.93%
Veff (total) = 61.946
k= 2.00 (@ 95 % level)
U(x) = 2.4
%U(x) = 3.86 %

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Method Precision	60.2889	0.507410	0.842 %
m_{zc}	0.04333	0.000083	0.1916 %
m_y	0.04578	0.000083	0.1813 %
m_{yc}	0.04662	0.000083	0.1781 %
m_x	0.09872	0.000083	0.0841 %
w_z	68.4482	0.530507	0.7750 %
R'b	1.1047	0.009552	0.8647 %
R'bc	1.1379	0.008094	0.7113 %
Sample preparation effect	1.000	0.0100	1.000 %
Recovery	1.000	0.0023	0.226 %

Uncertainty Information from PTB

Leucine	Phenylalanine
w_{Sample}^{exp} = Mass fraction of Leucine in solution per Aliquot, mean of 9 single observations à 11.5 % P_{Leucin} = Purity of the reference material à 1.2 % K_W = Uncertainty of weighing à 0.0 % S_{ys} = Estimated factor for unidentified systematic error à 87.3 % The uncertainty budget was calculated using the GUM workbench and is attached in the appendix.	w_{Sample}^{exp} = Mass fraction of Phenylalanin in solution per Aliquot, mean of 9 single observations à 11.0 % $P_{Phenyalanin}$ = Purity of the reference material à 7,9 % K_W = Uncertainty of weighing à 0.0 % S_{ys} = Estimated factor for unidentified systematic error à 81.1 % The uncertainty budget was calculated using the GUM workbench and is attached in the appendix.

Uncertainty Information from UME

Leucine

Uncertainty budget of Leucine			
	Value	u(x)	u(x)/x
Weighing of sample (mg)	25	4.29E-06	1.72E-07
Weighing of IS (mg)	25	1.29E-08	5.17E-10
Standard stock solution (mg/kg)	5000	1.13E+01	2.26E-03
Internal stock solution (mg/kg)	5000	1.52E+01	3.03E-03
Recovery	1	3.57E-02	3.57E-02
Repeatability	100	8.77E-02	8.77E-04
Calibration graph	1.5	5.87E-02	3.91E-02
			5.31E-02
Result (mg/kg) 19.73			
Combined uncertainty	1.05		
Expanded uncertainty	2.10		
% Relative uncertainty	10.62		
% Relative standard uncertainty	5.31		

Phenylalanine

Uncertainty budget of Phenylalanine			
	Value	u(x)	u(x)/x
Weighing of sample (mg)	25	4.29E-06	1.72E-07
Weighing of IS (mg)	25	1.29E-08	5.17E-10
Standard stock solution (mg/kg)	5000	1.13E+01	2.26E-03
Internal stock solution (mg/kg)	5000	1.53E+01	3.06E-03
Recovery	1	3.52E-02	3.52E-02
Repeatability	100	2.60E-01	2.60E-03
Calibration graph	0.68	2.87E-03	4.23E-03
			3.57E-02
Result (mg/kg) 57.65			
Combined uncertainty	2.06		
Expanded uncertainty	4.12		
% Relative uncertainty	7.14		
% Relative standard uncertainty	3.57		

Uncertainty Information from VNIIM

Leucine	Phenylalanine

$$U(x) = 2 \cdot u(x) \qquad \frac{u_{(x)}}{x} = \sqrt{\left(\frac{u_{(cal)}}{w_{cal}}\right)^2 + \left(\frac{u_{(w_x)}}{w_x}\right)^2 + \left(\frac{u_{(blank)}}{w_{blank}}\right)^2}$$

$$\frac{u_{(cal)}}{w_{cal}} = \sqrt{\left(\frac{u_{(m_{IS})}}{m_{IS}}\right)^2 + \sum\left(\frac{u_{(m_{nat})}}{m_{nat}}\right)^2 + \left(\frac{u_{(w_{pur})}}{w_{pur}}\right)^2 + \left(\frac{u_{(RF)}}{RF}\right)^2 + \left(\frac{u_{(RF_{av})}}{RF_{av}}\right)^2}$$

$$\frac{u_{(w_x)}}{w_x} = \sqrt{\left(\frac{u_{(m_{IS})}}{m_{IS}}\right)^2 + \left(\frac{u_{(m_x)}}{m_x}\right)^2 + \left(\frac{u_{(w_{alq})}}{w_{alq}}\right)^2 + \left(\frac{u_{(w_{smpl})}}{w_{smpl}}\right)^2}$$

$u_{(cal)}$ - the standard uncertainty of the calibration;
$u_{(w_x)}$ – the standard uncertainty of the mass fraction of analyte in the Sample
$u_{(blank)}$ - the standard uncertainty of the mass fraction of analyte in the Blank
$u_{(m_{IS})}$ - the standard uncertainty of the mass (preparation of the Internal Standard solution)
$u_{(m_{nat})}$ – the standard uncertainty of the mass (preparation of the native stock solution)
$u_{(w_{pur})}$ – the standard uncertainty of the purity of aminoacids
$u_{(RF_{av})}$ - the standard uncertainty of determination of average Response Factor (RF)
$u_{(RF)}$ - the standard uncertainty of adjustments of RF
$u_{(m_{IS})}$ - the standard uncertainty of the mass (addition the Internal Standard solution into the Sample)
$u_{(m_x)}$ - the standard uncertainty of the mass of the Sample (weighing the Sample)
$u_{(w_{alq})}$- the standard uncertainty of determination average mass fraction of aminoacids between aliquots
$u_{(w_{smpl})}$ - the standard uncertainty of determination average mass fraction of aminoacids between vials

	Type	Leu			Phe		
		m, mg	u(m), mg	u, %	m, mg	u(m), mg	u, %
$u_{(mIS)}$	B	11.3	0.007	0.062%	19.19	0.007	0.036%
		958.56	0.012	0.001%	968.11	0.012	0.001%
		129.5	0.009	0.007%	79.58	0.015	0.019%
		3899.8	0.032	0.001%	3846.16	0.032	0.001%
				0.06%			**0.04%**
$u_{(m_{nat})}$	B	15.42	0.007	0.045%	17.34	0.007	0.040%
		1004	0.016	0.002%	1006.51	0.016	0.002%
		19.67	0.007	0.036%	19.87	0.007	0.035%
		985.56	0.012	0.001%	987.72	0.012	0.001%
				0.06%			**0.05%**
$u_{(w_{pur})}$	B			**0.30%**			**0.30%**
$u_{(m_{cal})}$	B			**0.84%**			**0.40%**
$u_{(m_x)}$	B	250	0.009	0.004%	250	0.009	0.004%
$u_{(m_{IS})}$	B	18	0.007	0.039%	36	0.007	0.019%
$u_{(RF)}$	B			**0.84%**			**0.40%**
$u_{(RF_{av})}$	B			**0.74%**			**0.39%**
$u_{(w_{smpl})}$	A			**1.8%**			**1.4%**
$u_{(w_{alq})}$	A			**3.1%**			**2.4%**

APPENDIX H: Participants' Quantitative Results as Reported

The following are text excerpts and/or pictures of the quantitative results as provided by the participants in the reporting form. Information is grouped by participant and presented in alphabetized acronym order.

Quantitative Results from EXHM/GCSL-EIM

Plasma Results Leucine

Bottle Number	**8280/001/0026**	**8280/001/0291**	**8280/001/0522**
Result (mg/kg)	20.11	19.93	19.88
Overall Mean (mg/kg)	19.97		
Combined Standard Uncertainty (mg/kg)	0.87		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.74		

Plasma Results Phenylalanine

Bottle Number	**8280/001/0026**	**8280/001/0291**	**8280/001/0522**
Result (mg/kg)	59.63	59.44	60.15
Overall Mean (mg/kg)	59.74		
Combined Standard Uncertainty (mg/kg)	2.16		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	4.32		

Quantitative Results from HSA

Plasma Results Leucine

Bottle Number	0376	0776	0483
Result (mg/kg)	21.37	19.06	22.06
Overall Mean (mg/kg)			
Combined Standard Uncertainty (mg/kg)			
Coverage Factor, *k* (95 % confidence interval)			
Expanded uncertainty at 95 % confidence interval (mg/kg)			

Plasma Results Phenylalanine

Bottle Number	0376	0776	0483
Result (mg/kg)	61.16	60.00	61.29
Overall Mean (mg/kg)	60.81		
Combined Standard Uncertainty (mg/kg)	0.83		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.66		

Quantitative Results from INMETRO

Plasma Results Leucine

Bottle Number	**499**	**491**	**063**
Result (mg/kg)	21.53	20.91	18.59
Overall Mean (mg/kg)	20.3		
Combined Standard Uncertainty (mg/kg)	1.1		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	2.2		

Plasma Results Phenylalanine

Bottle Number	**499**	**491**	**063**
Result (mg/kg)	61.36	60.77	59.98
Overall Mean (mg/kg)	60.7		
Combined Standard Uncertainty (mg/kg)	1.3		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	2.6		

Quantitative Results from KRISS

Plasma Results Leucine

Bottle Number	#329	#851	#861
Result (mg/kg)	21.76	22.28	22.38
Overall Mean (mg/kg)	22.14		
Combined Standard Uncertainty (mg/kg)	0.27		
Coverage Factor, *k* (95 % confidence interval)	2.31		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.63		

Plasma Results Phenylalanine

Bottle Number	#329	#851	#861
Result (mg/kg)	61.35	61.83	61.25
Overall Mean (mg/kg)	61.48		
Combined Standard Uncertainty (mg/kg)	0.70		
Coverage Factor, k (95 % confidence interval)	2.20		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.53		

Quantitative Results from LGC

Plasma Results Leucine

Bottle Number	**716**	**421**	**45**
Result (mg/kg)	**19.58 ± 0.19**	**21.84 ± 0.31**	**19.22 ± 0.20**
Overall Mean (mg/kg)	**N/A**		
Combined Standard Uncertainty (mg/kg)	**N/A**		
Coverage Factor, *k* (95 % confidence interval)	**N/A**		
Expanded uncertainty at 95 % confidence interval (mg/kg)	**N/A**		

Plasma Results Phenylalanine

Bottle Number	**716**	**421**	**45**
Result (mg/kg)	59.26	60.57	58.93
Overall Mean (mg/kg)	59.59		
Combined Standard Uncertainty (mg/kg)	0.72		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.45		

Quantitative Results from NMIA

Plasma Results Leucine

Bottle Number	**264**	**825**	**307**
Result (mg/kg)	19.04	19.06	19.00
Overall Mean (mg/kg)	Not reported as requested		
Combined Standard Uncertainty (mg/kg)	0.23		
Coverage Factor, *k* (95 % confidence interval)	2.04		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.48		

Plasma Results Phenylalanine

Bottle Number	**264**	**825**	**307**
Result (mg/kg)	62.7	60.0	61.1
Overall Mean (mg/kg)	61.4		
Combined Standard Uncertainty (mg/kg)	0.88		
Coverage Factor, *k* (95 % confidence interval)	2.13		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.9		

Quantitative Results from NMIJ / AIST

Plasma Results Leucine

Bottle Number	0297	0535	0977
Result (mg/kg)	19.1	19.2	19.0
Overall Mean (mg/kg)	19.1		
Combined Standard Uncertainty (mg/kg)	0.15		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.30		

Plasma Results Phenylalanine

Bottle Number	0297	0535	0977
Result (mg/kg)	60.2	60.2	60.0
Overall Mean (mg/kg)	60.1		
Combined Standard Uncertainty (mg/kg)	0.32		
Coverage Factor, k (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.64		

Quantitative Results from NMIT

Plasma Results Leucine

Bottle Number	LGC8280/001/0953	LGC8280/001/0552	LGC8280/001/0907
Result (mg/kg)	**19.34**	**19.28**	**19.28**
Overall Mean (mg/kg)	19.28		
Combined Standard Uncertainty (mg/kg)	0.44		
Coverage Factor, *k* (95 % confidence interval)	1.99		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.87		

Plasma Results Phenylalanine

Bottle Number	LGC8280/001/0953	LGC8280/001/0552	LGC8280/001/0907
Result (mg/kg)	60.0	60.4	60.3
Overall Mean (mg/kg)	60.3		
Combined Standard Uncertainty (mg/kg)	1.2		
Coverage Factor, *k* (95 % confidence interval)	2.00		
Expanded uncertainty at 95 % confidence interval (mg/kg)	2.4		

Quantitative Results from PTB

Plasma Results Leucine

Bottle Number	LGC 8280/011/0751	LGC 8280/011/0938	LGC 8280/011/0810
Result (mg/kg)	19.48 mg/kg	19.42 mg/kg	19.37 mg/kg
Overall Mean (mg/kg)	19.42 mg/kg		
Combined Standard Uncertainty (mg/kg)	0.21 mg/kg		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	0.42 mg/kg		

Plasma Results Phenylalanine

Bottle Number	LGC 8280/011/0751	LGC 8280/011/0938	LGC 8280/011/0810
Result (mg/kg)	60.77 mg/kg	60.95 mg/kg	59.62 mg/kg
Overall Mean (mg/kg)	60.45 mg/kg		
Combined Standard Uncertainty (mg/kg)	0.67 mg/kg		
Coverage Factor, k (95% confidence interval)	2		
Expanded uncertainty at 95% confidence interval (mg/kg)	1.3 mg/kg		

Quantitative Results from UME

Plasma Results Leucine

Bottle Number	**986**	**558**	**364**
Result (mg/kg)	19.73	19.67	21.58
Overall Mean (mg/kg)			
Combined Standard Uncertainty (mg/kg)	1.05	1.04	1.15
Coverage Factor, k (95 % confidence interval)	2	2	2
Expanded uncertainty at 95 % confidence interval (mg/kg)	2.10	2.09	2.29

Plasma Results Phenylalanine

Bottle Number	**986**	**558**	**364**
Result (mg/kg)	57.39	57.35	58.20
Overall Mean (mg/kg)	57.65		
Combined Standard Uncertainty (mg/kg)	2.06		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	4.12		

Quantitative Results from VNIIM

Plasma Results Leucine

Bottle Number	LGC8280/001/0681	LGC8280/001/0760	LGC8280/001/0736
Result (mg/kg)	19.72	19.31	18.19
Overall Mean (mg/kg)	19.1		
Combined Standard Uncertainty (mg/kg)	0.7		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	1.5		

Plasma Results Phenylalanine

Bottle Number	LGC8280/001/0681	LGC8280/001/0760	LGC8280/001/0736
Result (mg/kg)	57.11	58.93	58.45
Overall Mean (mg/kg)	58.5		
Combined Standard Uncertainty (mg/kg)	1.7		
Coverage Factor, *k* (95 % confidence interval)	2		
Expanded uncertainty at 95 % confidence interval (mg/kg)	3.4		